

60 Rec'd PCT/PTO 26 JUL 2001

Form PTO-1390

U.S. DEPARTMENT OF COMMERCE  
PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

P21289

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371U.S. APPLICATION NO. (If known, see 37 CFR  
1.5)

69/869334

INTERNATIONAL APPLICATION NO.

PCT/JP00/00472

INTERNATIONAL FILING DATE

28 January 2000

PRIORITY DATE CLAIMED

29 January 1999

TITLE OF INVENTION

PROCESS FOR PRODUCING HHMG-CoA REDUCTASE INHIBITORS

APPLICANT(S) FOR DO/EO/US

Hirofumi ENDO, Yoshiyuki YONETANI, Hiroshi MIZOGUCHI, Shin-ichi HASHIMOTO, and Akio OZAKI

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (U.S.C. 371(c)(5)).

Items 11 to 16 below concern other document(s) or information included:

11. Assignee: KYOWA HAKKO KOGYO CO., Ltd. Of Tokyo, JAPAN
12. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
13. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
14. ☒ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ Figure of Drawing to be published \_\_\_\_\_
18. ☒ Other items or information:
  - Cover Sheet and International Application as published(in Japanese).
  - Computer-readable copy of Sequence Listing.
  - Paper Copy of Sequence Listing.
  - PCT/RO/101-PCT Request(in English and Japanese).
  - PCT/IPEA/416(in Japanese).
  - PCT/IPEA/409(in Japanese).
  - PCT/IPEA/408(in Japanese).
  - PCT/IB/301.
  - PCT/IB/304.
  - PCT/IB/308.
  - PCT/IB/332.
  - PCT/ISA/210(in English and Japanese).
  - Cover Letter under 35 USC 371 and 1.495.
  - Claim of Priority.

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/869334

INTERNATIONAL APPLICATION NO.

PCT/JP00/00472

ATTORNEY'S DOCKET NUMBER

P21289

19. The following fees are submitted:

CALCULATIONS

PTO USE ONLY

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Search report has been prepared by the EPO or JPO. . . . . \$ 860.00

International preliminary examination fee paid to USPTO (37 CFR 1.482). . . . . \$ 690.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO(37 CFR 1.445(a)(2)). . . . . \$ 710.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO. . . . . \$1,000.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4). . . . . \$ 100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

\$860.00

Surcharge of \$130.00 for furnishing the oath or declaration later than 20 30 months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

Claims

Number Filed

Number Extra

RATE

Total Claims

39

- 20 =

19

X \$18.00

\$342.00

Independent Claims

8

- 3 =

5

X \$80.00

\$400.00

Multiple dependent claim(s) (if applicable)

+ \$270.00

\$

TOTAL OF ABOVE CALCULATIONS =

\$1602.00

Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.

\$

SUBTOTAL =

\$1602.00

Processing fee of \$130.00 for furnishing the English translation later than 20 30 months from the earliest claimed priority date (37 CFR 1.492(f)).

+

Extension of Time fee in the amount of \$

TOTAL NATIONAL FEE =

\$1602.00

Fee for recording the enclosed assignment (37 CFR 1.21(h). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property

+

TOTAL FEES ENCLOSED =

\$1602.00

Amount to be refunded

\$

Charged

\$

a. ☒ A check in the amount of \$1602.00 to cover the above fees is enclosed.b. ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_ to cover the above fees.c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0089.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO CUSTOMER NO. 7055

AT THE PRESENT ADDRESS OF:

Bruce H. Bernstein  
GREENBLUM & BERNSTEIN, P.L.C.  
1941 Roland Clarke Place  
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SIGNATURE

Bruce H. Bernstein

NAME

33094

29,027

REGISTRATION NUMBER

P21289.A01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Hirofumi ENDO et al.  
Serial No : Not Yet Assigned (National Stage of PCT/JP00/00472)  
Filed : Concurrently Herewith (International Filing Date January 28, 2000)  
For : PROCESS FOR PRODUCING HMG-CoA REDUCTASE INHIBITORS

**PRELIMINARY AMENDMENT**

Commissioner of Patents and Trademarks  
Washington, D.C. 20231

Sir:

Prior to calculation of the filing fees and the examination of the above-identified patent application on the merits, the Examiner is respectfully requested to amend the claims as follows:

IN THE CLAIMS

Please amend the claims as follows (a marked-up copy of the claim amendments is provided as an attachment to this Amendment):

5. (Amended-Clean Text) The protein according to claim 1, wherein the microorganism belonging to the genus *Baccillus* is a microorganism selected from *B. subtilis*, *B. megaterium*, *B. laterosporus*, *B. sphaericus*, *B. pumilus*, *B. stearothermophilus*, *B. cereus*, *B. badius*, *B. brevis*, *B. alvei*, *B. circulans* and *B. macerans*.

6. (Amended-Clean Text) The protein according to claim 1, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis* ATCC6051, *B. megaterium* ATCC10778, *B. megaterium* ATCC11562, *B. megaterium* ATCC13402, *B. megaterium* ATCC15177, *B. megaterium* ATCC15450, *B. megaterium* ATCC19213, *B. megaterium* IAM1032, *B. laterosporus* ATCC4517, *B. pumilus* FERM BP-2064, *B. badius* ATCC14574, *B. brevis* NRRL B-8029, *B. alvei* ATCC6344, *B. circulans* NTCT-2610, and *B. macerans* NCIMB-9368.

7.(Amended-Clean Text) The protein according to claim 1, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *Bacillus* sp. FERM BP-6029 or *Bacillus* sp. FERM BP-6030.

17. (Amended-Clean Text) An isolated DNA encoding the protein according to claim 1.

21. (Amended-Clean Text) A recombinant DNA vector comprising the DNA according to claim 14.

24. (Amended-Clean Text) The transformant according to claim 22, wherein the transformant belongs to microorganism selected from *Escherichia coli*, *Bacillus subtilis*, *Bacillus megaterium*, *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Corynebacterium callunae* and *Streptomyces lividans*.

25. (Amended-Clean Text) A process for producing compound (II-a) or compound (II-b), wherein the transformant according to claim 22, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

- allowing compound (I-a) or compound (I-b) to exist in an aqueous medium;
- allowing compound (II-a) or compound (II-b) to be produced and accumulated in said aqueous medium; and
- collecting compound (II-a) or compound (II-b) from said aqueous medium.

26. (Amended-Clean Text) A process for producing compound (IV-a) or compound (IV-b), wherein the transformant according to claim 22, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

- allowing compound (III-a) or compound (III-b) to exist in an aqueous medium;
- allowing compound (IV-a) or compound (IV-b) to be produced and accumulated in said aqueous medium; and

collecting compound (IV-a) or compound (IV-b) from said aqueous medium.

27. (Amended-Clean Text) A process for producing compound (VI-a) or compound (VI-b), wherein the transformant according to claim 22, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:  
allowing compound (V-a) or compound (V-b) to exist in an aqueous medium;  
allowing compound (VI-a) or compound (VI-b) to be produced and accumulated in said aqueous medium; and  
collecting compound (VI-a) or compound (VI-b) from said aqueous medium.

28. (Amended-Clean Text) A process for producing compound (VIII-a) or compound (VIII-b), wherein the transformant according to claim 22, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:  
allowing compound (VII-a) or compound (VII-b) to exist in an aqueous medium;  
allowing compound (VIII-a) or compound (VIII-b) to be produced and accumulated in said aqueous medium; and  
collecting compound (VIII-a) or compound (VIII-b) from said aqueous medium.

37. (Amended-Clean Text) The process according to claim 25, wherein the treated

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product of the culture of the transformant is a treated product selected from cultured cells; treated products such as dried cells, freeze-dried cells, cells treated with a surfactant, cells treated with an enzyme, cells treated by ultrasonication, cells treated by mechanical milling, cells treated by solvent; a protein fraction of a cell; and an immobilized products of cells or treated cells.

38. (Amended) A process for producing a protein, which comprises culturing a transformant obtained by introducing a recombinant DNA vector comprising the DNA having the nucleotide sequence shown by SEQ ID NO: 2 in a medium; producing and accumulating the protein according to claim 1 in the culture; and collecting said protein from said culture.

#### REMARKS

By the above amendment, the claims have been amended to delete multiple dependency.

If there should be any questions, the Examiner is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,  
Hirofumi ENDO et al.

Bruce H. Bernstein  
Reg. No. 29,027

July 24 2001

33,094

P21289.A01

GREENBLUM & BERNSTEIN, P.L.C.  
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MARKED-UP COPY OF AMENDED CLAIMS

5. (Amended) The protein according to claim 1 [any one of claims 1 to 4], wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis*, *B. megaterium*, *B. laterosporus*, *B. sphaericus*, *B. pumilus*, *B. stearothermophilus*, *B. cereus*, *B. badius*, *B. brevis*, *B. alvei*, *B. circulans* and *B. macerans*.

6. (Amended) The protein according to claim 1 [any one of claims 1 to 5], wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis* ATCC6051, *B. megaterium* ATCC10778, *B. megaterium* ATCC11562, *B. megaterium* ATCC13402, *B. megaterium* ATCC15177, *B. megaterium* ATCC15450, *B. megaterium* ATCC19213, *B. megaterium* IAM1032, *B. laterosporus* ATCC4517, *B. pumilus* FERM BP-2064, *B. badius* ATCC14574, *B. brevis* NRRL B-8029, *B. alvei* ATCC6344, *B. circulans* NTCT-2610, and *B. macerans* NCIMB-9368.

7.(Amended) The protein according to claim 1 [any one of claims 1 to 5], wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *Bacillus* *sp.* FERM BP-6029 or *Bacillus* *sp.* FERM BP-6030.

17. (Amended) An isolated DNA encoding the protein according to claim 1 [any one of claims 1 to 12].

21. (Amended) A recombinant DNA vector comprising the DNA according to claim 14 [any one of claims 14 to 20].

24. (Amended) The transformant according to claim 22 [or 23], wherein the transformant belongs to microorganism selected from *Escherichia coli*, *Bacillus subtilis*, *Bacillus megaterium*, *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Corynebacterium callunae* and *Streptomyces lividans*.

25. (Amended) A process for producing compound (II-a) or compound (II-b), wherein the transformant according to claim 22 [any one of claims 22 to 24], a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

allowing compound (I-a) or compound (I-b) to exist in an aqueous medium;

allowing compound (II-a) or compound (II-b) to be produced and accumulated in said aqueous medium; and

collecting compound (II-a) or compound (II-b) from said aqueous medium.

26. (Amended) A process for producing compound (IV-a) or compound (IV-b), wherein the transformant according to claim 22 [any one of claims 22 to 24], a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

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allowing compound (III-a) or compound (III-b) to exist in an aqueous medium;

allowing compound (IV-a) or compound (IV-b) to be produced and accumulated in said aqueous medium; and

collecting compound (IV-a) or compound (IV-b) from said aqueous medium.

27. (Amended) A process for producing compound (VI-a) or compound (VI-b), wherein the transformant according to claim 22 [any one of claims 22 to 24], a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

allowing compound (V-a) or compound (V-b) to exist in an aqueous medium;

allowing compound (VI-a) or compound (VI-b) to be produced and accumulated in said aqueous medium; and

collecting compound (VI-a) or compound (VI-b) from said aqueous medium.

28. (Amended) A process for producing compound (VIII-a) or compound (VIII-b), wherein the transformant according to claim 22 [any one of claims 22 to 24], a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

allowing compound (VII-a) or compound (VII-b) to exist in an aqueous medium;

allowing compound (VIII-a) or compound (VIII-b) to be produced and accumulated in said aqueous medium; and

collecting compound (VIII-a) or compound (VIII-b) from said aqueous medium.

37. (Amended) The process according to claim 25 [any one of claims 25 to 28], wherein the treated product of the culture of the transformant is a treated product selected from cultured cells; treated products such as dried cells, freeze-dried cells, cells treated with a surfactant, cells treated with an enzyme, cells treated by ultrasonication, cells treated by mechanical milling, cells treated by solvent; a protein fraction of a cell; and an immobilized products of cells or treated cells.

38. (Amended) A process for producing a protein, which comprises culturing a [the] transformant obtained by introducing a recombinant DNA vector comprising the DNA having the nucleotide sequence shown by SEQ ID NO: 2 [according to any one of claims 22 to 24] in a medium; producing and accumulating the protein according to claim 1 [any one of claims 1 to 12] in the culture; and collecting said protein from said culture.

# SPECIFICATION

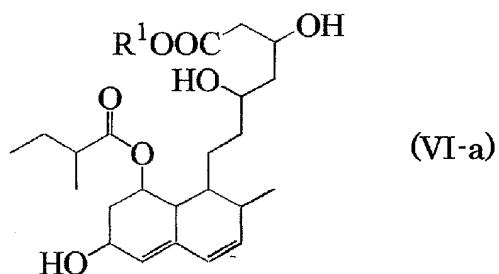
## PROCESS FOR PRODUCING HMG-CoA REDUCTASE INHIBITORS

### Technical Field

The present invention relates to a DNA which is related to the production of a compound which inhibits hydroxymethylglutaryl CoA (HMG-CoA) reductase and has an action of reducing serum cholesterol, and a process for producing said compound using the DNA.

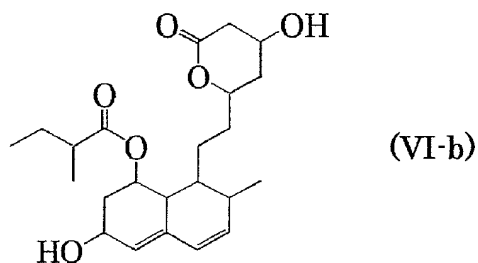
### Background Art

A compound represented by the formula (VI-a) (hereinafter referred to as compound (VI-a)):



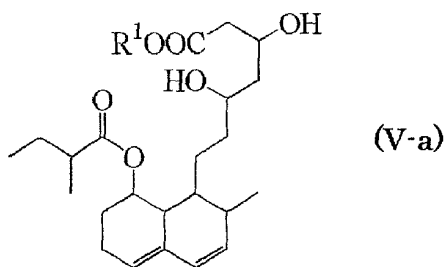
wherein  $R^1$  represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal; or

a lactone form of compound (VI-a) represented by the formula (VI-b) (hereinafter referred to as compound (VI-b)):

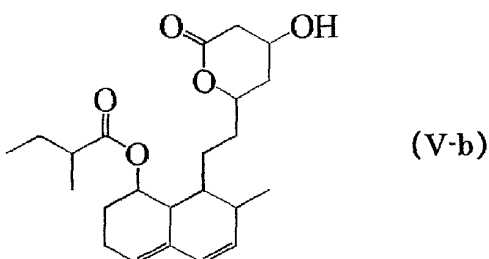


is known to inhibit HMG-CoA reductase and exhibit an action of reducing serum cholesterol (*The Journal of Antibiotics*, 29, 1346 (1976)).

There have been several reports regarding methods for producing compound (VI-a) or compound (VI-b) from a compound represented by the formula (V-a) (hereinafter referred to as compound (V-a)):



wherein  $R^1$  has the same definition as the above; or from the lactone form of compound (V-a) represented by the formula (V-b) (hereinafter referred to as compound (V-b)):



using a microorganism.

Specifically, Japanese Patent Application Laid-Open (kokai) No. 57-50894 describes a method which uses filamentous fungi; both Japanese Patent Application Laid-Open (kokai) No. 7-184670 and International Publication WO96/40863 describe a method which uses *Actinomycetes*; and Japanese Patent No. 2672551 describes a method which uses recombinant *Actinomycetes*. As is well known, however, since filamentous fungi and *Actinomycetes* grow with filamentous form by elongating hyphae, the viscosity of the culture in a fermentor increases.

This often causes a shortage of oxygen in the culture, and since the culture becomes heterogeneous, reaction efficiency tends to be reduced. In order to resolve this oxygen shortage and maintain homogeneity of the culture, the agitation rate of the fermentor should be raised, but by raising the agitation rate, hyphae are sheared and, as a result, activity of the microorganisms tends to decrease (Basic Fermentation Engineering (Hakko Kogaku no Kiso) p.169-190, P.F. Stansbury, A. Whitaker, Japan Scientific Societies Press (1988)).

#### Disclosure of the Invention

The object of the present invention is to provide a DNA encoding a novel hydroxylase, and an industrially advantageous method for producing a compound which inhibits HMG-CoA reductase and has an action of reducing the level of serum cholesterol.

The present inventors considered that, if the hydroxylation of compound (I-a) or compound (I-b) could be carried out with a microorganism forming no hyphae, inconvenience such as the decrease of reaction efficiency due to the heterogeneity of the culture caused by hyphae formation could be avoided, and that this would be industrially advantageous. Thus, as a result of intensive studies, the present inventors have accomplished the present invention.

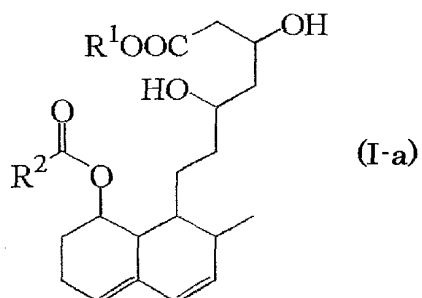
Thus, the present invention relates to the following (1) to (39).

Hereinafter, in the formulas,  $R^1$  represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal, and  $R^2$  represents a substituted or unsubstituted alkyl, or a substituted or unsubstituted aryl, unless otherwise specified.

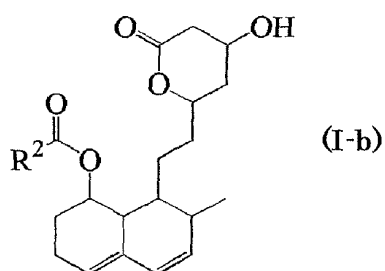
- (1) A protein which is derived from a microorganism belonging to the genus *Bacillus*,

and has an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b),

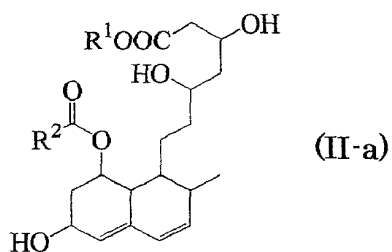
wherein the compound (I-a) is a compound represented by the formula (I-a) :



the compound (I-b) is a lactone form of compound (I-a) and is represented by the formula (I-b):

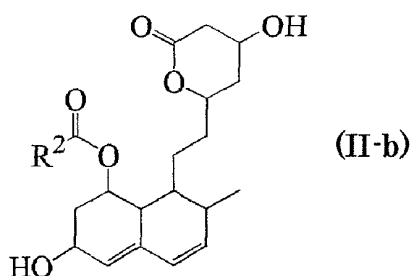


the compound (II-a) is a compound represented by the formula (II-a):



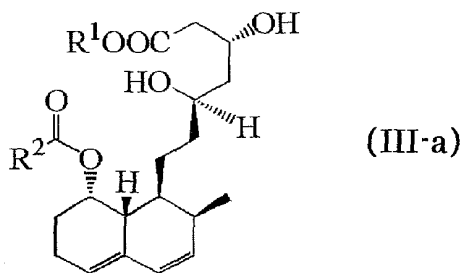


the compound (II-b) is a lactone form of compound (II-a) and is represented by the formula (II-b):

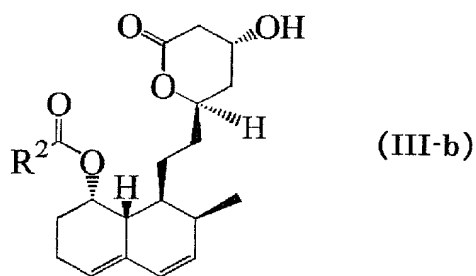


(2) A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (IV-a) or compound (IV-b) from compound (III-a) or compound (III-b),

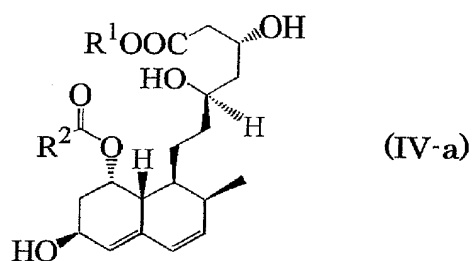
wherein the compound (III-a) is a compound represented by the formula (III-a) :



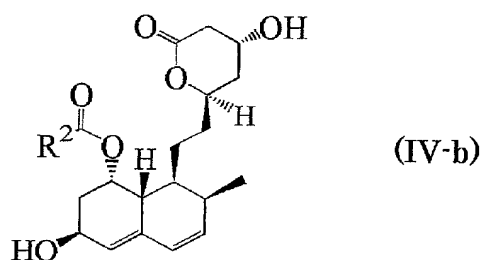
the compound (III-b) is a lactone form of compound (III-a) and is represented by the formula (III-b):



the compound (IV-a) is a compound represented by the formula (IV-a):

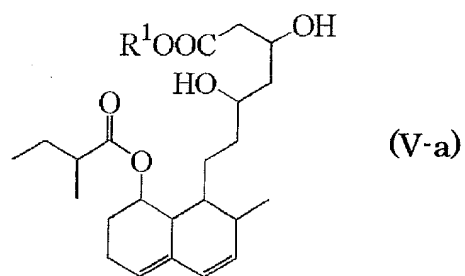


the compound (IV-b) is a lactone form of compound (IV-a) and is represented by the formula (IV-b):

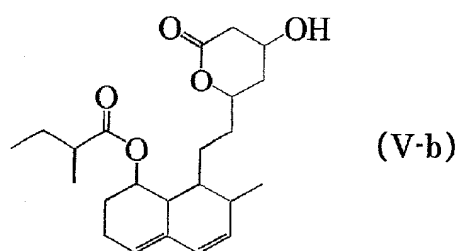


(3) A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (VI-a) or compound (VI-b) from compound (V-a) or compound (V-b),

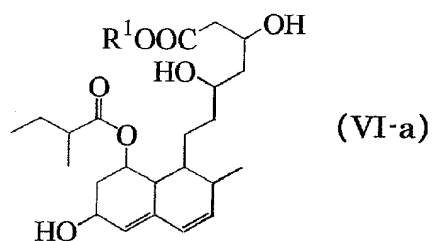
wherein the compound (V-a) is a compound represented by the formula (V-a) :



the compound (V-b) is a lactone form of compound (V-a) and is represented by the formula (V-b):

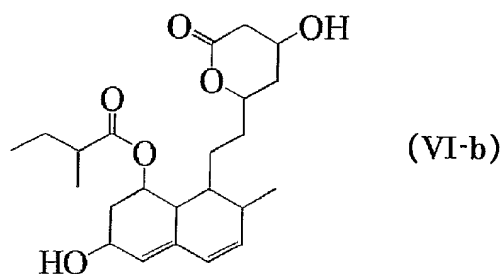


the compound (VI-a) is a compound represented by the formula (VI-a):



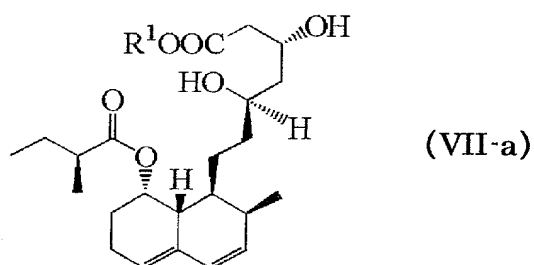
; and

the compound (VI-b) is a lactone form of compound (VI-a) and is represented by the formula (VI-b):

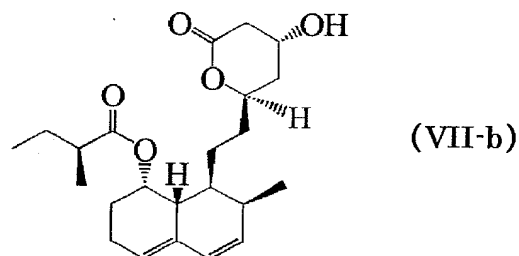


(4) A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (VIII-a) or compound (VIII-b) from compound (VII-a) or compound (VII-b),

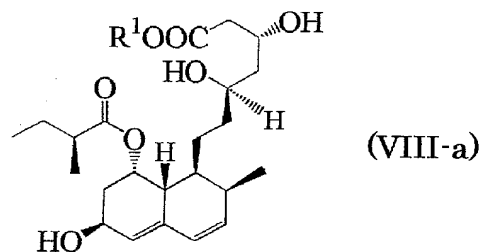
wherein the compound (VII-a) is a compound represented by the formula (VII-a):



the compound (VII-b) is a lactone form of compound (VII-a) and is represented by the formula (VII-b):

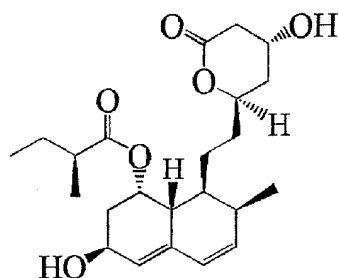


the compound (VIII-a) is a compound represented by the formula (VIII-a):



; and

the compound (VIII-b) is a lactone form of compound (VIII-a) and is represented by the formula (VIII-b):



(VIII-b)

(5) The protein according to any one of (1) to (4) above, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis*, *B. megaterium*, *B. laterosporus*, *B. sphaericus*, *B. pumilus*, *B. stearothermophilus*, *B. cereus*, *B. badius*, *B. brevis*, *B. alvei*, *B. circulans* and *B. macerans*.

(6) The protein according to any one of (1) to (5) above, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis* ATCC6051, *B. megaterium* ATCC10778, *B. megaterium* ATCC11562, *B. megaterium* ATCC13402, *B. megaterium* ATCC15177, *B. megaterium* ATCC15450, *B. megaterium* ATCC19213, *B. megaterium* IAM1032, *B. laterosporus* ATCC4517, *B. pumilus* FERM BP-2064, *B. badius* ATCC14574, *B. brevis* NRRL B-8029, *B. alvei* ATCC6344, *B. circulans* NTCT-2610, and *B. macerans* NCIMB-9368.

(7) The protein according to any one of (1) to (5) above, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *Bacillus* sp. FERM BP-6029 or *Bacillus* sp. FERM BP-6030.

(8) A protein having the amino acid sequence shown by SEQ ID NO: 1.

(9) A protein which has an amino acid sequence comprising deletion, substitution or addition of one or more amino acids in the amino acid sequence shown by SEQ ID NO:

1, and has an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b).

(10) The protein according to (9) above, wherein the protein has the amino acid sequence shown by SEQ ID NO: 42 or 45.

(11) The protein according to (9) above, wherein the compound (I-a) is compound (III-a), the compound (I-b) is compound (III-b), the compound (II-a) is compound (IV-a), and the compound (II-b) is compound (IV-b).

(12) The protein according to (9) above, wherein the compound (I-a) is compound (V-a), the compound (I-b) is compound (V-b), the compound (II-a) is compound (VI-a), and the compound (II-b) is compound (VI-b).

(13) The protein according to (9) above, wherein the compound (I-a) is compound (VII-a), the compound (I-b) is compound (VII-b), the compound (II-a) is compound (VIII-a), and the compound (II-b) is compound (VIII-b).

(14) An isolated DNA having the nucleotide sequence shown by SEQ ID NO: 2.

(15) An isolated DNA which hybridizes with the DNA according to (14) above under stringent conditions, and encodes a protein having an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b).

(16) The DNA according to (15) above, wherein the DNA has a nucleotide sequence selected from the group consisting of the nucleotide sequences shown by SEQ ID NOS: 41, 43 and 44.

(17) An isolated DNA encoding the protein according to any one of (1) to (12) above.

(18) The DNA according to (15) above, wherein the compound (I-a) is compound (III-a), the compound (I-b) is compound (III-b), the compound (II-a) is compound (IV-a), and the compound (II-b) is compound (IV-b).

(19) The DNA according to (15) above, wherein the compound (I-a) is compound (V-a), the compound (I-b) is compound (V-b), the compound (II-a) is compound (VI-a), and the compound (II-b) is compound (VI-b).

(20) The DNA according to (15) above, wherein the compound (I-a) is compound (VII-a), the compound (I-b) is compound (VII-b), the compound (II-a) is compound (VIII-a), and the compound (II-b) is compound (VIII-b).

(21) A recombinant DNA vector comprising the DNA according to any one of (14) to (20) above.

(22) A transformant obtained by introducing the recombinant DNA vector according to (21) above into a host cell.

(23) The transformant according to (22) above, wherein the transformant belongs to a microorganism selected from the genera *Escherichia*, *Bacillus*, *Corynebacterium*, and *Streptomyces*.

(24) The transformant according to (22) or (23) above, wherein the transformant belongs to microorganism selected from *Escherichia coli*, *Bacillus subtilis*, *Bacillus megaterium*, *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Corynebacterium callunae* and *Streptomyces lividans*.

(25) A process for producing compound (II-a) or compound (II-b), wherein the

transformant according to any one of (22) to (24) above, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

allowing compound (I-a) or compound (I-b) to exist in an aqueous medium;

allowing compound (II-a) or compound (II-b) to be produced and accumulated in said aqueous medium; and

collecting compound (II-a) or compound (II-b) from said aqueous medium.

(26) A process for producing compound (IV-a) or compound (IV-b), wherein the transformant according to any one of (22) to (24) above, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

allowing compound (III-a) or compound (III-b) to exist in an aqueous medium;

allowing compound (IV-a) or compound (IV-b) to be produced and accumulated in said aqueous medium; and

collecting compound (IV-a) or compound (IV-b) from said aqueous medium.

(27) A process for producing compound (VI-a) or compound (VI-b), wherein the transformant according to any one of (22) to (24) above, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:

allowing compound (V-a) or compound (V-b) to exist in an aqueous medium;

allowing compound (VI-a) or compound (VI-b) to be produced and accumulated in said aqueous medium; and

collecting compound (VI-a) or compound (VI-b) from said aqueous medium.

(28) A process for producing compound (VIII-a) or compound (VIII-b), wherein the transformant according to any one of (22) to (24) above, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process



comprises:

allowing compound (VII-a) or compound (VII-b) to exist in an aqueous medium;  
allowing compound (VIII-a) or compound (VIII-b) to be produced and accumulated in said aqueous medium; and  
collecting compound (VIII-a) or compound (VIII-b) from said aqueous medium.

(29) The process according to (25) above, wherein the compound (II-b) is the compound (II-b) obtained by forming a lacton from compound (II-a).

(30) The process according to (25) above, wherein the compound (II-a) is the compound (II-a) obtained by opening the lactone ring of compound (II-b).

(31) The process according to (26) above, wherein the compound (IV-b) is the compound (IV-b) obtained by forming a lacton from compound (IV-a).

(32) The process according to (26) above, wherein the compound (IV-a) is the compound (IV-a) obtained by opening the lactone ring of compound (IV-b).

(33) The process according to (27) above, wherein the compound (VI-b) is the compound (VI-b) obtained by forming a lacton from compound (VI-a).

(34) The process according to (27) above, wherein the compound (VI-a) is the compound (VI-a) obtained by opening the lactone ring of compound (VI-b).

(35) The process according to (28) above, wherein the compound (VIII-b) is the compound (VIII-b) obtained by forming a lacton from compound (VIII-a).

(36) The process according to (28) above, wherein the compound (VIII-a) is the compound (VIII-a) obtained by opening the lactone ring of compound (VIII-b).

(37) The process according to any one of (25) to (28) above, wherein the treated product of the culture of the transformant is a treated product selected from cultured cells; treated products such as dried cells, freeze-dried cells, cells treated with a surfactant, cells treated with an enzyme, cells treated by ultrasonication, cells treated by mechanical milling, cells treated by solvent; a protein fraction of a cell; and an immobilized products of cells or treated cells.

(38) A process for producing a protein, which comprises culturing the transformant according to any one of (22) to (24) above in a medium; producing and accumulating the protein according to any one of (1) to (12) above in the culture; and collecting said protein from said culture.

(39) An oligonucleotide corresponding to a sequence consisting of 5 to 60 continuous nucleotides in a nucleotide sequence selected from the group consisting of the nucleotide sequences shown by SEQ ID NOS: 2, 41, 43 and 44; or an oligonucleotide corresponding to a complementary sequence to said oligonucleotide.

The present invention will be described in detail below.

#### 1. Obtaining of yjiB gene

The DNA of the present invention can be obtained by PCR method [Science, 230, 1350 (1985)] using the genome nucleotide sequence information of a chromosome of *Bacillus subtilis* which has already been determined [<http://www.pasteur.fr/Bio/SubtiList.html>] and the information on *Bacillus subtilis* yjiB gene deduced from said genome nucleotide sequence.

Specifically, the DNA of the present invention can be obtained by the following method.

*Bacillus subtilis* (e.g., *B. subtilis* ATCC15563) is cultured by a usual manner in

a medium suitable for *Bacillus subtilis*, e.g. LB liquid medium [containing Bacto Trypton (produced by Difco) 10g, yeast extract (produced by Difco) 5g, and NaCl 5g in 1L of water; and adjusted to pH 7.2]. After culturing, the cells are collected from the culture by centrifugation.

A chromosomal DNA is isolated from the collected cells by a known method (e.g., Molecular Cloning 2<sup>nd</sup> ed).

Using the nucleotide sequence information shown by SEQ ID NO:2, sense and antisense primers containing nucleotide sequences corresponding to the DNA region encoding a protein of the present invention are synthesized with a DNA synthesizer.

After amplification by PCR, in order to enable introduction of said amplified DNA fragments into a plasmid, it is preferred that an appropriate restriction site such as BamHI, EcoRI or the like is added at 5' end of the sense and antisense primers.

Examples of combinations of said sense and antisense primers include combination of DNAs having nucleotide sequences shown by SEQ ID NOS:13 and 14.

Using chromosomal DNA as a template, PCR is performed with these primers, TaKaRa LA-PCR<sup>TM</sup> Kit Ver. 2 (TaKaRa), Expand<sup>TM</sup> High-Fidelity PCR System (Boehringer Mannheim) or the like by a DNA Thermal Cycler (Perkin-Elmer Japan).

When PCR is performed, for example, the following method can be carried out. In the case where the above primer is a DNA fragment of 2kb or less, each cycle consists of reaction steps of 30 seconds at 94°C, 30 seconds to 1 minute at 55°C, and 2 minutes at 72°C. In the case where the above primer is a DNA fragment of more than 2kb, each cycle consists of reaction steps of 20 seconds at 98°C and 3 minutes at 68°C. In any case, PCR is performed under conditions where the 30 cycles are repeated, and then reaction is carried out for 7 minutes at 72°C.

The amplified DNA fragments are cut at the same restriction site as the site which is formed using the above primers, and then the DNA fragments are fractioned and recovered by a method such as agarose gel electrophoresis, sucrose density gradient ultracentrifugation and the like.

Using the recovered DNA fragments, a cloning vector is produced by a usual

method such as methods described in Molecular Cloning 2<sup>nd</sup> ed., Current Protocols in Molecular Biology, Supplement 1-38, John Wiley & Sons (1987-1997) (abbreviated as Current Protocols in Molecular Biology, Supplement hereinafter), DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University Press (1995), or by using a commercially available kit such as SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (produced by Life Technologies), ZAP-cDNA Synthesis Kit (produced by Stratagene), etc., then the thus-produced cloning vector is used to transform *Escherichia coli*, e.g. *E. coli* DH5  $\alpha$  strain (available from TOYOBO).

Examples of a cloning vector for the transformation of *E. coli* include a phage vector and plasmid vector insofar as it is capable of self-replicating in *E. coli* K12 strain. An expression vector for *E. coli* can also be used as a cloning vector. Specifically, examples thereof include ZAP Express [produced by Stratagene, Strategies, 5, 58 (1992)], pBluescript II SK(+) [Nucleic Acids Research, 17, 9494 (1989)], Lambda ZAP II (produced by Stratagene),  $\lambda$ gt10,  $\lambda$ gt11 [DNA Cloning, A Practical Approach, 1, 49 (1985)],  $\lambda$ TriplEx (produced by Clontech),  $\lambda$ ExCell (produced by Pharmacia), pT7T318U (produced by Pharmacia), pcD2 [H.Okayama and P.Berg ; Mol. Cell. Biol., 3, 280 (1983)], pMW218 (produced by Wako Pure Chemical Industries), pUC118, pSTV28 (produced by Takara), pEG400 [J. Bac., 172, 2392 (1990)], pHMV1520 (produced by MoBiTec), pQE-30 (produced by QIAGEN), etc.

A plasmid containing a desired DNA can be obtained from the obtained transformed strain by usual methods described in e.g. Molecular Cloning 2<sup>nd</sup> edition, Current Protocols in Molecular Biology Supplement, DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, and Oxford University Press (1995), etc.

Using the aforementioned method, a plasmid containing a DNA encoding a protein which catalyzes reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b), can be obtained.

Examples of the plasmids include the below-mentioned pSyjiB.

Apart from the aforementioned method, a plasmid containing a DNA encoding a protein which catalyzes a reaction of producing compound (II-a) or compound (II-b)

from compound (I-a) or compound (I-b) can be obtained also by a method wherein a chromosomal library of *Bacillus subtilis* is prepared with a suitable vector using *E. coli* as a host, and the activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b) is measured on each strain of this library.

The nucleotide sequence of the above-obtained gene can be used to obtain homologues of the DNA from other prokaryotes or plants in the same manner as mentioned above.

The DNA and DNA fragment of the present invention obtained in the above method can be used to prepare oligonucleotides such as antisense oligonucleotides, sense oligonucleotides etc. having a partial sequence of the DNA of the present invention or such oligonucleotides containing RNAs. Alternatively, based on the sequence information of the above-obtained DNA, these oligonucleotids can be synthesized with the above DNA synthesizer.

Examples of the oligonucleotides include a DNA having the same sequence as a contiguous 5 to 60 nucleotides in the nucleotide sequence of the above DNA, or a DNA having a complementary sequence to said DNA. RNAs having complementary sequences to these DNAs are also oligonucleotides of the present invention.

Examples of said oligonucleotides include a DNA having the same sequence as a contiguous 5 to 60 nucleotides sequence in the nucleotide sequences shown by SEQ ID NOS:2, 41, 43 or 44, or a DNA having a complementary sequence to said DNA. If these are used as sense and antisense primers, the aforementioned oligonucleotides without extreme difference in melting temperatures ( $T_m$ ) and numbers of bases are preferably used. Specifically, examples thereof include oligonucleotides having a nucleotide sequence shown by SEQ ID NOS: 3 to 39.

Furthermore, derivatives of these oligonucleotides (referred to as oligonucleotide derivative hereinafter) can also be used as the DNA of the present invention.

Oligonucleotide derivatives include a oligonucleotide derivative whose phosphate diester linkage is replaced by a phosphorothioate linkage, an oligonucleotide

derivative whose phosphate diester linkage is replaced by a N3'-P5' phosphoamidate linkage, an oligonucleotide derivative whose ribose and phosphate diester linkage is replaced by a peptide-nucleic acid linkage, an oligonucleotide derivative whose uracil is replaced by C-5 propinyl uracil, an oligonucleotide derivative whose uracil is replaced by C-5 thiazol uracil, an oligonucleotide derivative whose cytosine is replaced by C-5 propinyl cytosine, an oligonucleotide derivative whose cytosine is replaced by phenoxazine-modified cytosine, an oligonucleotide derivative whose ribose is replaced by 2'-O-propyl ribose, or an oligonucleotide derivative whose ribose is replaced by 2'-methoxy-ethoxyribose, etc. [Saibo Kogaku, 16, 1463 (1997).]

## II. Method for producing a protein which catalyzes a reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b)

In order to express the above-obtained DNA in a host cell, the desired DNA fragment is cut into a fragment of suitable length containing said gene using restriction enzymes or DNase enzymes, followed by inserting the fragment into a site downstream of a promoter in an expression vector, and then the expression vector is introduced into host cells suitable for use of the expression vector.

The host cells may be any of bacteria, yeasts, animal cells, insect cells or the like insofar as they can express the objective gene.

As an expression vector, a vector capable of being autonomously replicated in a host cell or capable of being integrated into a chromosome, and containing a promoter at a site suitable for transcription of the above objective gene, is used.

When prokaryotes such as bacteria are used as the host cell, the expression vector for expressing the above DNA is preferably a vector autonomously replicable in said cell and is a recombinant vector composed of a promoter, a ribosome-binding sequence, the above DNA and a transcription termination sequence. A gene for regulating the promoter may be contained.

The expression vectors include pBTrp2, pBTac1, pBTac2 (all of which are commercially available from Boehringer Mannheim), pKK233-2 (produced by

Pharmacia), pSE280 (produced by Invitrogen), pGEMEX-1 (produced by Promega), pQE-8 (produced by QIAGEN), pQE-30 (produced by QIAGEN), pKYP10 (Japanese Patent Application Laid-Open No. 58-110600), pKYP200 [Agricultural Biological Chemistry, 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci., USA, 82, 4306 (1985)], pBluescriptII SK(+), pBluescriptII SK(-) (produced by Stratagene), pTrS30 (FERM BP-5407), pTrS32 (FERM BP-5408), pGEX (produced by Pharmacia), pET-3 (produced by Novagen), pTerm2 (US 4,686,191, US 4,939,094, US 5,160,735), pSupex, pUB110, pTP5, pC194, pUC18 [gene, 33, 103 (1985)], pUC19 [Gene, 33, 103 (1985)], pSTV28 (produced by Takara), pSTV29 (produced by Takara), pUC118 (produced by Takara), pPA1 (Japanese Patent Application Laid-Open No. 63-233798), pEG400 [J. Bacteriol., 172, 2392 (1990)], pQE-30 (produced by QIAGEN), PHY300 (produced by Takara), pHW1520 (produced by MoBiTec), etc.

The promoter may be any one insofar as it can be expressed in a host cell. Examples are promoters derived from *E.coli*, phage etc., such as trp promoter (P<sub>trp</sub>), lac promoter (P<sub>lac</sub>), PL promoter, PR promoter and PSE promoter, and SP01 promoter, SP02 promoter, penP promoter and the like. Artificially designed and modified promoters such as a P<sub>trp</sub>×2 promoter having two P<sub>trp</sub> promoters in tandem, tac promoter, letI promoter, and lacT7 promoter can also be used. Furthermore, xylA promoter for expression in *Bacillus* bacteria or P54-6 promoter for expression in *Corynebacterium* bacteria can also be used.

Any ribosome binding sequences may be used insofar as they can work in a host cell, and a plasmid in which the distance between a Shine-Dalgarno sequence and an initiation codon is adjusted to an appropriate distance (for example, 6 to 18 bases) may be preferably used.

For efficient transcription and translation, a protein which catalyzes the reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b) wherein the N-terminus or a part thereof is deleted may be fused to the N-terminus part of a protein encoded by the expression vector, and the thus-obtained

fused protein may be expressed. Such examples include the below-mentioned pWyjiB.

Although a transcription termination sequence is not necessarily required for expression of the desired DNA, it is preferred to locate the transcription termination sequence just downstream from the structural gene.

Examples of prokaryotes include microorganisms belonging to the genus *Escherichia*, *Corynebacterium*, *Brevibacterium*, *Bacillus*, *Microbacterium*, *Serratia*, *Pseudomonas*, *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Arthrobacter*, *Azotobacter*, *Chromatium*, *Erwinia*, *Methylobacterium*, *Phormidium*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Streptomyces*, *Synechococcus*, and *Zymomonas*, preferably *Escherichia*, *Corynebacterium*, *Brevibacterium*, *Bacillus*, *Pseudomonas*, *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Arthrobacter*, *Azotobacter*, *Chromatium*, *Erwinia*, *Methylobacterium*, *Phormidium*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Streptomyces*, *Synechococcus*, and *Zymomonas*.

Specific examples of the microorganisms include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* DH5  $\alpha$ , *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No.49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Escherichia coli* MP347, *Escherichia coli* NM522, *Bacillus subtilis* ATCC33712, *Bacillus megaterium*, *Bacillus* sp. FERM BP-6030, *Bacillus amyloliquefaciens*, *Brevibacterium ammoniagenes*, *Brevibacterium immariophilum* ATCC14068, *Brevibacterium saccharolyticum* ATCC14066, *Brevibacterium flavum* ATCC14067, *Brevibacterium lactofermentum* ATCC13869, *Corynebacterium glutamicum* ATCC13032, *Corynebacterium glutamicum* ATCC14297, *Corynebacterium acetoacidophilum* ATCC13870, *Corynebacterium callunae* ATCC15991, *Microbacterium ammoniophilum* ATCC15354, *Serratia ficaria*, *Serratia fonticola*, *Serratia liquefaciens*, *Serratia marcescens*, *Pseudomonas* sp. D-0110, *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, *Agrobacterium rubi*, *Anabaena cylindrical*, *Anabaena doliolum*, *Anabaena flos-aquae*, *Arthrobacter aureus*, *Arthrobacter citreus*, *Arthrobacter globiformis*, *Arthrobacter hydrocarboglutamicus*,



*Arthrobacter mysorens*, *Arthrobacter nicotianae*, *Arthrobacter paraffineus*, *Arthrobacter protophormiae*, *Arthrobacter roseoparaffinus*, *Arthrobacter sulfurous*, *Arthrobacter ureafaciens*, *Chromatium buderi*, *Chromatium tepidum*, *Chromatium vinosum*, *Chromatium warmingii*, *Chromatium fluviatile*, *Erwinia uredovora*, *Erwinia carotovora*, *Erwinia ananas*, *Erwinia herbicola*, *Erwinia punctata*, *Erwinia terreus*, *Methylobacterium rhodesianum*, *Methylobacterium extorquens*, *Phormidium* sp. ATCC29409, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodopseudomonas blastica*, *Rhodopseudomonas marina*, *Rhodopseudomonas palustris*, *Rhodospirillum rubrum*, *Rhodospirillum salexigens*, *Rhodospirillum salinarum*, *Streptomyces ambofaciens*, *Streptomyces aureofaciens*, *Streptomyces aureus*, *Streptomyces fungicidicus*, *Streptomyces griseochromogenes*, *Streptomyces griseus*, *Streptomyces lividans*, *Streptomyces olivogriseus*, *Streptomyces rameus*, *Streptomyces tanashiensis*, *Streptomyces vinaceus*, and *Zymomonas mobilis*.

The method for introducing the recombinant vector may be any method for introducing DNA into the host cells described above. For examples, mention can be made of a method using calcium ions [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], a protoplast method (Japanese Patent Application Laid-Open No. 63-248394), an electroporation method, a method described in Gene, 17, 107 (1982) and Molecular & General Genetics, 168, 111 (1979), and the like.

If yeasts are used as the host cell, expression vectors such as YEp13 (ATCC37115), YEp24 (ATCC37051), YCp50 (ATCC37419), pHS19, and pHS15 can be exemplified.

Any promoter can be used insofar as they can be expressed in yeasts. For example, mention can be made of promoters such as PHO5 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock protein promoter, MF $\alpha$ 1 promoter, and CUP 1 promoter.

Examples of host cells include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans*, and *Schwanniomyces alluvius*.

The method for introducing a recombinant vector may be any method for introducing DNA into yeast, and examples include an electroporation method [Methods Enzymol., 194, 182 (1990)], a spheroplast method [Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)], a lithium acetate method [J. Bacteriol., 153, 163 (1983)] and a method describe in Proc. Natl. Acad. Sci. USA, 75, 1929 (1978).

If animal cells are used as the host cells, expression vectors such as pcDNA1, pcDM8 (commercially available from Funakoshi), pAGE107 (Japanese Patent Application Laid-Open No. 3-22979; Cytotechnology, 3, 133 (1990)), pAS3-3 (Japanese Patent Application Laid-Open No. 2-227075), pCDM8 [Nature, 329, 840 (1987)], pcDNA1/Amp (Invitrogen), pREP4 (Invitrogen), pAGE103 [J. Biochem., 101, 1307 (1987)], and pAGE210 can be used.

The promoter to be used may be any promoter which can be expressed in animal cells. Examples are a promoter for IE (immediate early) gene of cytomegalovirus (human CMV), SV40 early promoter, retrovirus promoter, metallothionein promoter, heat shock promoter, Sr  $\alpha$  promoter and the like. Furthermore, an enhancer of the IE gene of human CMV may be used together with a promoter.

Examples of animal cells include Namalwa cell, HBT5637 (Japanese Patent Application Laid-Open No. 63-299), COS1 cell, COS7 cell, CHO cell and the like.

The method for introducing a recombinant vector into animal cells may be any method for introducing DNA into animal cells. Examples of such methods include an electroporation method [Cytotechnology, 3, 133 (1990)], a calcium phosphate method (Japanese Patent Application Laid-Open No. 2-227075), a lipofection method [Proc. Natl. Acad. Sci., USA, 84, 7413 (1987)], a method described in Virology, 52, 456 (1973), and the like. Obtaining and culturing of the transformant can be conducted according to methods described in Japanese Patent Application Laid-Open No. 2-227075 or Japanese Patent Application Laid-Open No. 2-257891.

If insect cells are used as the host cells, the protein can be expressed by methods described in Baculovirus Expression Vectors, A Laboratory Manual, Current

Protocols in Molecular Biology Supplement 1-38 (1987-1997); Bio/Technology, 6, 47 (1988) and the like.

That is, a recombinant gene transfer vector and a baculovirus are co-transfected into insect cells to obtain a recombinant virus in the culture supernatant of the insect cells, and then the insect cells are infected with the recombinant virus whereby the protein can be expressed.

Examples of the gene transfer vectors used in this method include pVL1392, pVL1393 and pBlueBacIII (all manufactured by Invitrogen).

As the baculovirus, it is possible to employ, e.g. Autographa californica nuclear polyhedrosis virus, that is, a virus infecting insects of the family Barathra.

As the insect cells, it is possible to use Sf9, Sf21 [Baculovirus Expression Vectors, A Laboratory Manual, W.H. Freeman and Company, New York (1992)] which are oocytes of *Spodopetera frugiperda* and High 5 (Invitrogen) which is oocyte of *Trichoplusia ni*, and the like.

As a method for co-transferring the aforesaid recombinant gene transfer vector and the aforesaid baculovirus into insect cells for preparing the recombinant virus, for example, a calcium phosphate method (Japanese Patent Application Laid-Open No. 2-227075), a lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)] and the like may be used.

As a method for expressing gene, in addition to direct expression, secretory production, expression of a fusion protein and the like can be carried out according to the method described in Molecular Cloning 2<sup>nd</sup> edition.

When a protein has been expressed by yeasts, animal cells or insect cells, the protein to which a sugar or sugar chain is added can be obtained.

The thus-obtained transformant is cultured in a medium to produce and accumulate proteins which catalyze the reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b) in the culture, and the proteins are recovered from the culture, thereby producing the protein which catalyzes production of compound (II-a) or compound (II-b) from compound (I-a) or compound

(I-b).

As a method for culturing in a medium the transformant for the production of the protein of the present invention which catalyzes the reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b), conventional methods used for culturing a transformant in a host cell can be used.

If the transformant of the present invention is a prokaryote such as *E.coli* or an eukaryote such as yeast, the medium for culturing these organisms may be either a natural or synthetic medium insofar as it contains a carbon source, a nitrogen source, inorganic salts and the like which can be assimilated by said organisms, and it allows efficient culturing of the transformant.

As a carbon source, any carbon source can be used as long as it can be assimilated by the microorganisms, including carbohydrates such as glucose, fructose, sucrose, or molasses containing those sources, starch or starch hydrolysates; organic acids such as acetic acid, propionic acid; and alcohols such as ethanol, propanol.

As a nitrogen source, the following can be used: ammonia; ammonium salts of various inorganic acids and organic acids, such as ammonium chloride, ammonium sulfate, ammonium acetate, and ammonium phosphate; other nitrogen-containing compounds; and peptone, meat extracts, yeast extracts, corn steep liquor, casein hydrolysates, soy bean meal, soy bean meal hydrolysates, various fermented cells and hydrolysates thereof, and the like.

Examples of the inorganic substances include potassium dihydrogenphosphate, potassium hydrogenphosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate and calcium carbonate.

The culturing is carried out under aerobic conditions by shake culturing or aeration-agitation culturing or the like. The culturing temperature is preferably 15 to 50°C, and the culturing period is usually 16 hours to 7 days. While culturing, pH is maintained at 3.0 to 9.0. The pH control is conducted using an inorganic or organic acid, alkaline solution, urea, calcium carbonate, ammonia and the like.

If necessary, antibiotics such as ampicillin and tetracycline may be added to the

medium while culturing.

When a microorganism transformed with an expression vector using an inductive promoter as a promoter is cultured, an inducer may be optionally added to the medium. For example, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), indole acrylic acid (IAA) or xylose may be added to the medium respectively, when a microorganism transformed with expression vectors containing lac promoter, trp promoter, or xylA promoter is used.

The medium for culturing the transformant obtained by using animal cells as host cells may be a generally-used medium such as RPMI1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle's MEM medium [Science, 122, 501 (1952)], DMEM medium [Virology, 8, 396 (1959)], 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)] or any one of these media further supplemented with fetal calf serum.

Culturing is usually carried out for 1 to 7 days at pH 6 to 8 at 30 to 40°C in the presence of 5% CO<sub>2</sub>.

If necessary, antibiotics such as kanamycin and penicillin may be added to the medium while culturing.

The medium for culturing the transformant obtained by using insect cells as host cells may be a generally-used medium such as TNM-FH medium (produced by Pharmingen), Sf-900 II SFM medium (produced by Gibco BRL), ExCell 400 and ExCell 405 [both are products of JRH Biosciences], Grace's Insect Medium [Grace, T.C.C., Nature, 195, 788 (1962)] or the like.

Culturing is usually carried out at pH 6 to 7 at a temperature of 25 to 30°C for a period of 1 to 5 days.

If necessary, antibiotics such as gentamycin may be added to the medium while culturing.

For isolating and purifying the protein which catalyzes a reaction of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b) from the culture of the transformant of the present invention, any conventional methods for the

isolation and purification of enzymes can be performed.

For example, in the case where the protein of the present invention is expressed in a soluble form in cells, after culturing, the cells are recovered by centrifugation and suspended in an aqueous buffer, followed by disruption with ultrasonic disrupter, French Press, Manton-Gaulin homogenizer, Dynomill or the like, thereby obtaining a cell-free extract. From the supernatant obtained by centrifuging the cell-free extract, a purified preparation can be obtained by using conventional methods for isolation and purification of enzymes alone or in combination, such as solvent extraction, salting-out or desalting with sulfate ammonium etc., precipitation with an organic solvent, anion-exchange chromatography on resin such as diethylaminoethyl (DEAE)-Sephadex, DIAION HPA-75 (produced by Mitsubishi Chemical Industries Ltd.) or the like, cation-exchange chromatography on resin such as S-Sephadex FF (Pharmacia) or the like, hydrophobic chromatography on resin such as butyl Sephadex, phenyl Sephadex or the like, gel filtration using molecular sieve, affinity chromatography, chromatofocusing, and electrophoresis such as isoelectric electrophoresis.

In the case where the protein is expressed in a form of an inclusion body in cells, the cells are similarly recovered, disrupted and centrifuged, thereby obtaining a precipitated fraction, and the protein is recovered from the fraction in a usual manner. The recovered inclusion body is solubilized with a protein denaturing agent. The solubilized solution is then diluted with or dialyzed against a solution not containing the protein denaturing agent or a solution containing the protein denaturing agent at a concentration low enough not to denature the protein, whereby the protein is renatured to have normal tertiary structure, and its purified preparation can be obtained by the same isolation and purification method as described above.

When the protein of the present invention or a saccharide modified derivatives thereof are extracellularly secreted, the protein or the derivatives to which saccharide chain is added, can be recovered from the supernatant of the culture. That is, the culture is subjected to an above-mentioned process such as centrifugation and the like, thereby obtaining soluble fractions, then a purified preparation can be obtained from

said soluble fractions in the same manner as in the above.

Examples of the thus-obtained proteins include proteins having amino acid sequences shown by SEQ ID NOS: 1, 42 or 45. Furthermore, the protein expressed in the above manner can also be produced by chemically synthesis methods such as Fmoc method (fluorenyl methyloxycarbonyl method) and tBoc method (t-butyloxycarbonyl method). Alternatively, the protein can be obtained by synthesis using a peptide synthesizer manufactured by Sowa Trading (Advanced chemTech, USA), Perkin-Elmer Japan (Perkin Elmer, USA), Pharmacia Biotech (Pharmacia Biotech, Sweden), ALOKA (Protein Technology Instrument, USA), KURABO (Synthecell-Vega, USA), Japan PerSeptive Ltd. (PerSeptive, USA), Shimazu, etc.

### III. Production of compound (II-a) or compound (II-b)

Using cells obtained by culturing the transformant obtained in above II according to the method described in above II, a culture of said cells, a treated product of said culture, or an enzyme extracted from said cells as enzyme sources, compound (II-a) or compound (II-b) can be produced by allowing compound (I-a) or compound (I-b) to exist in an aqueous medium, allowing compound (II-a) or compound (II-b) to be produced and accumulated in the above aqueous medium, and collecting compound (II-a) or compound (II-b) from the above aqueous medium.

Examples of treated products of the culture of the cells include the treated products of the cells such as dried cells, lyophilized cells, cells treated with surfactants, cells treated with enzymes, cells treated with ultrasonication, cells treated with mechanical milling, cells treated with solvents; or protein fractions of the cells; or immobilized products of said cell and said treated products of said cells.

As a method for converting compound (I-a) or compound (I-b) into compound (II-a) or compound (II-b), both of the following methods (a) and (b) can be used: (a) a method wherein the compound (I-a) or compound (I-b) is previously added to the medium for culturing cells; and (b) a method wherein compound (I-a) or compound (I-b) is added to the medium while culturing. Alternatively, a method wherein the

enzyme source obtained from the cell culture is reacted with compound (I-a) or compound (I-b) in the aqueous medium can be also used.

In a case where compound (I-a) or compound (I-b) is added to a medium in which a microorganism is to be cultured, 0.1 to 10mg, preferably 0.2 to 1mg of compound (I-a) or compound (I-b) is added to 1 ml of medium at the beginning of or at some midpoint of the culture. It is desired that compound (I-a) or compound (I-b) is added after it is dissolved in an organic solvent such as methyl alcohol or ethyl alcohol.

In a case where a method of allowing an enzyme source obtained by culturing cells to act upon compound (I-a) or compound (I-b) in an aqueous medium, the amount of enzyme to be used depends on the specific activity of the enzyme source or the like. For example, when a culture of cells, cells, or a treated product thereof is used as an enzyme source, 5 to 1,000mg, preferably 10 to 400mg of enzyme source is added per 1mg of compound (I-a) or compound (I-b). The reaction is performed in an aqueous medium preferably at 20 to 50°C, and particularly preferably at 25 to 37°C. The reaction period depends on the amount, specific activity and the like of an enzyme source to be used, and it is usually 2 to 150 hours, preferably 6 to 120 hours.

Examples of an aqueous medium include water, or buffers such as phosphate buffer, HEPES (N-2 hydroxyethylpiperazine-N-ethanesulfonate) buffer and Tris (tris(hydroxymethyl)aminomethane)hydrochloride buffer. An organic solvent may be added to the above buffers, unless it inhibits reaction. Examples of organic solvent include acetone, ethyl acetate, dimethyl sulfoxide, xylene, methyl alcohol, ethyl alcohol and butanol. A mixture of an organic solvent and an aqueous medium is preferably used, for example when compound (I-b) is used.

In the case where compound (I-a) or compound (I-b) is added to the aqueous medium, compound (I-a) or compound (I-b) is dissolved in an aqueous medium capable of dissolving compound (I-a) or compound (I-b), and then is added to the medium. An organic solvent may be added to the above buffers, unless it inhibits reaction. Examples of organic solvents include acetone, ethyl acetate, dimethyl sulfoxide, xylene, methyl alcohol, ethyl alcohol and butanol.



Compound (I-b) and compound (II-b) can easily be converted into compound (I-a) and compound (II-a) respectively by a method for opening a lactone ring as mentioned below. Likewise, compound (I-a) and compound (II-a) can easily be converted into compound (I-b) and compound (II-b) respectively by a method for producing lactone as mentioned below.

Examples of a method for opening a lactone ring include a method which comprises dissolving compound (I-b) or compound (II-b) in an aqueous medium and adding thereto an acid or alkali. Examples of the aqueous medium include water and an aqueous solution containing salts, which does not inhibit the reaction, such as phosphate buffer, Tris buffer and the like. The above aqueous solution may contain an organic solvent such as methanol, ethanol, ethyl acetate and the like in a concentration which does not inhibit the reaction. Examples of acid include acetic acid, hydrochloric acid and sulfuric acid, and examples of alkali include sodium hydroxide, potassium hydroxide and ammonia.

Examples of a method for producing lactone include a method which comprises dissolving compound (I-a) or compound (II-a) in a non-aqueous solvent and adding thereto an acid or base catalyst. As long as the non-aqueous solvent is an organic solvent which does not substantially contain water and can dissolve compound (I-a) or compound (II-a), any type of non-aqueous solvent can be used. Examples of non-aqueous solvents include dichloromethane and ethyl acetate. As a catalyst, any catalyst can be used, as long as it catalyzes lactonization and does not show any actions other than lactonization on a substrate or a reaction product. Examples of the above catalyst include trifluoroacetic acid and para-toluenesulfonic acid. Reaction temperature is not particularly limited, but is preferably 0 to 100°C, and is more preferably 20 to 80°C.

The collection of compound (II-a) or compound (II-b) from the reaction solution can be carried out by any ordinary methods used in the field of organic synthetic chemistry such as extraction with organic solvents, crystallization, thin-layer chromatography, high performance liquid chromatography, and the like.

As a method for detecting and quantifying compound (II-a) or compound (II-b) obtained by the present invention, any method can be used, as long as the detection or quantification of compound (II-a) and/or compound (II-b) can be performed. Examples thereof include  $^{13}\text{C}$ -NMR spectroscopy,  $^1\text{H}$ -NMR spectroscopy, mass spectroscopy and high performance liquid chromatography (HPLC).

In the present invention, some compounds of compound (I-a), compound (I-b), compound (II-a) and compound (II-b) can have stereoisomers such as optical isomers. The present invention covers all possible isomers and mixtures thereof including these stereoisomers.

As compound (I-a), compound (III-a) is preferable, compound (V-a) is more preferable, and compound (VII-a) is particularly preferable.

As compound (I-b), compound (III-b) is preferable, compound (V-b) is more preferable, and compound (VII-b) is particularly preferable.

As compound (II-a), compound (IV-a) is preferable, compound (VI-a) is more preferable, and compound (VIII-a) is particularly preferable.

As compound (II-b), compound (IV-b) is preferable, compound (VI-b) is more preferable, and compound (VIII-b) is particularly preferable.

Alkyl is a linear or branched alkyl containing 1 to 10 carbon atoms, preferably 1 to 6 carbon atoms, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, neopentyl, hexyl, isohexyl, heptyl, 4,4-dimethylpentyl, octyl, 2,2,4-trimethylpentyl, nonyl, decyl, and various branched chain isomers thereof.

Examples of aryl include phenyl and naphthyl.

The substituent in the substituted alkyl may be 1 to 3 identical or different groups, and examples thereof include halogens, hydroxy, amino, alkoxy and aryl.

The substituent in the substituted aryl may be 1 to 3 identical or different groups, and examples thereof include halogens, hydroxy, amino, alkyl and alkoxy.

The alkyl moiety in alkoxy has the same definition as in the alkyl mentioned above.

Alkali metal represents each element of lithium, sodium, potassium, rubidium,

cesium or francium.

The examples of the present invention is described below, but the present invention is not limited to these examples.

#### BEST MODE FOR CARRYING-OUT OF THE INVENTION

Example 1: Obtaining of the DNA encoding the protein having an activity of producing compound (VIII-a) or compound (VIII-b) from compound (VII-a) or compound (VII-b)

100mg of compound (VII-b) (produced by Sigma) was dissolved in 9.5ml of methanol, and 0.5ml of 1mol/l sodium hydroxide was added. The mixture was stirred at room temperature for 1 hour. The obtained reaction solution was dried to be solidified, and was dissolved by adding 5ml of deionized water, followed by adjusting pH to about 7 with about 0.1ml of 1mol/l hydrochloric acid. Then, 4.9ml of deionized water was added to the mixture to obtain 10ml of compound (VII-a), whose final concentration was 10mg/ml (a compound wherein, in formula (VII-a), R<sup>1</sup> is sodium).

*Bacillus subtilis* Marburg168 strain (ATCC15563) was inoculated with 1 platinum loop in a 10ml LB liquid medium, and cultured at 30°C overnight. After culturing, cells were collected from the obtained culture solution by centrifugation.

A chromosomal DNA was isolated and purified from the cells in a usual manner.

Sense and antisense primers having a combination of nucleotide sequences: SEQ ID NOS: 3 and 4, SEQ ID NOS: 5 and 6, SEQ ID NOS: 7 and 8, SEQ ID NOS: 9 and 10, SEQ ID NOS: 11 and 12, SEQ ID NOS: 13 and 14, and SEQ ID NOS: 15 and 16, were synthesized with a DNA synthesizer.

Using the chromosomal DNA as a template, PCR was performed with these primers and with TaKaRa LA-PCR<sup>TM</sup> Kit Ver.2 (produced by TAKARA), Expand<sup>TM</sup> High-Fidelity PCR System (produced by Boehringer Mannheim) or Taq DNA polymerase (produced by Boelinnger) using a DNA Thermal Cycler (produced by Perkin-Elmer Japan).

PCR was performed for 30 cycles in which each cycle consists of reaction steps of 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 72°C for DNA fragments of 2kb or less; and 20 seconds at 98°C, 3 minutes at 68°C for DNA fragments of more than 2kb, and then reaction was carried out for 7min at 72°C.

Among DNA fragments amplified by PCR, the DNA fragment (containing bioI gene) amplified by a combination of primers of SEQ ID NOS:3 and 4 was digested with restriction enzymes EcoRI and Sall, DNA fragment (containing cypA gene) amplified by a combination of primers of SEQ ID NOS:5 and 6 was digested with XbaI and SmaI, DNA fragment (containing cypX gene) amplified by a combination of primers of SEQ ID NOS:7 and 8 was digested with SmaI and Sall, DNA fragment (containing pksS gene) amplified by a combination of primers of SEQ ID NOS:9 and 10 was digested with EcoRI and Sall, DNA fragment (containing yet0 gene) amplified by a combination of primers of SEQ ID NOS:11 and 12 was digested with XbaI and BglII, DNA fragment (containing yjiB gene) amplified by a combination of primers of SEQ ID NOS:13 and 14 was digested with XbaI and SmaI, and DNA fragment (containing yrhJ gene) amplified by a combination of primers of SEQ ID NOS:15 and 16 was digested with XbaI and SmaI, respectively.

After digestion, the DNA fragments treated with the restriction enzymes were subjected to agarose gel electrophoresis to obtain the DNA fragments treated with various restriction enzymes.

A vector plasmid pUC119 (produced by TAKARA) was digested with restriction enzymes Sall and EcoRI, then subjected to agarose gel electrophoresis to obtain a Sall-EcoRI treated pUC119 fragment. Similarly, a vector plasmid pUC119 was digested with restriction enzymes Sall and SmaI, then subjected to agarose gel electrophoresis to obtain a Sall-SmaI treated pUC119 fragment.

pSTV28 (produced by TAKARA) was digested with restriction enzymes XbaI and SmaI, then subjected to agarose gel electrophoresis to obtain a XbaI-SmaI treated pSTV28 fragment. Similarly, a vector plasmid pSTV28 was digested with restriction enzymes XbaI and BamHI, then subjected to agarose gel electrophoresis to obtain a

XbaI-BamHI treated pSTV28 fragment.

The thus-obtained EcoRI-SalI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:3 and 4) was mixed with the SalI-EcoRI treated pUC119 fragment, XbaI-SmaI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:5 and 6) was mixed with the XbaI-SmaI treated pSTV28 fragment, SmaI-SalI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:7 and 8) was mixed with the SalI-SmaI treated pUC119 fragment, EcoRI-SalI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:9 and 10) was mixed with the SalI-EcoRI treated pUC119 fragment, XbaI-BglII treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:11 and 12) was mixed with the XbaI-BamHI treated pSTV28 fragment, XbaI-SmaI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:13 and 14) was mixed with the XbaI-SmaI treated pSTV28 fragment, XbaI-SmaI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:15 and 16) was mixed with XbaI-SmaI treated pSTV28 fragment, respectively. After ethanol precipitation, the obtained DNA precipitates were dissolved in 5  $\mu$ l of distilled water, and a ligation reaction was carried out to obtain each recombinant DNA.

Using the recombinant DNA, *E. coli* (purchased from TOYOBO) DH5  $\alpha$  strain is transformed by a usual method, then the transformant was plated to a LB agar medium [containing Bacto Trypton (produced by Difco) 10g, Bactoyeast extract (produced by Difco) 5g, NaCl 5g in 1L; and adjusted to pH7.4 with 1mol/l NaOH such that the agar is adjusted to 1.5%] containing 100  $\mu$ g/ml ampicillin in the case where the pUC119 is used as a vector plasmid; and to a LB agar medium containing 25  $\mu$ g/ml chloramphenicol in the case where the pSTV28 is used as a vector plasmid, followed by culturing for 2 days at 25°C.

Several colonies of the grown ampicillin-resistant or chloramphenicol-resistant transformants were selected, inoculated in 10ml LB liquid medium [which contains Bacto Trypton (produced by Difco) 10g, Bactoyeast extract (produced by Difco) 10g

and NaCl 5g in 1L; and is adjusted to pH 7.4 with 1mol/l NaOH], and then cultured while shaking for 2 days at 25°C.

The obtained culture was centrifuged to recover cells.

A plasmid was isolated from the cells in a usual manner.

The structure of the isolated plasmid was examined by cleaving it with various restriction enzymes and the nucleotide sequences were determined, thereby confirming that the desired DNA fragment was inserted in the plasmid. The plasmids obtained by linking the DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:3 and 4) treated with EcoRI-SalI to pUC119 fragment treated with SalI-EcoRI, was named pUbioI, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:5 and 6) treated with XbaI-SmaI to pSTV28 fragment treated with XbaI-SmaI was named pScypA, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:7 and 8) treated with SmaI-SalI to pUC119 fragment treated with SalI-SmaI was named pUcypX, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:9 and 10) treated with EcoRI-SalI to pUC119 fragment treated with SalI-EcoRI was named pUpksS, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:11 and 12) treated with XbaI-BglIII to pSTV28 fragment treated with XbaI-BamHI was named pSyet0, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:13 and 14) treated with XbaI-SmaI to pSTV28 fragment treated with XbaI-SmaI was named pSyjiB, the plasmids obtained by linking DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:15 and 16) treated with XbaI-SmaI to pSTV28 fragment treated with XbaI-SmaI was named pSyrhJ, respectively.

*E.coli* DH5  $\alpha$  containing the thus-obtained plasmid, *E.coli* DH5  $\alpha$  containing pUC119 or pSTV28, and *E.coli* DH5  $\alpha$  containing no plasmid were inoculated respectively in 3ml of LB liquid medium (to which a drug which corresponds to a drug-resistant gene in a vector plasmid was added) and cultured while shaking for 12

hours at 28°C. The culture solution (0.5ml) was inoculated to a LB liquid medium (to which a drug which corresponds to a drug-resistant gene was added) containing 1% glucose and 1% CaCO<sub>3</sub>, and was cultured while shaking for 12 hours at 28°C. The culture solution (1ml) was poured into an assist tube (produced by ASSIST), then glucose and the previously obtained compound (VII-a) (wherein R<sup>1</sup> is a Na) were added to a final concentration of 1% and 100mg/l, respectively, followed by shaking for 24 hours at 28°C. Upon completion of the reaction, cells were removed by centrifugation, then the obtained supernatant was thoroughly shaken with addition of the same amount of ethyl acetate. The upper ethyl acetate layer was separated from the solution by centrifugation, then the ethyl acetate layer was evaporated to dryness by a centrifugal evaporator. The dried matter was dissolved in one-fifths volume of methanol relative to that of the first culture supernatant, and subjected to a HPLC analysis [column; Inertsil ODS-2 (5μm, 4x250mm, manufactured by GL science), column temperature; 60 °C, mobile phase; acetonitrile: water: phosphoric acid=55:45:0.05, flow rate: 0.9ml/min, detection wavelength: 237nm] to detect and quantify the compound (VIII-a) (wherein R<sup>1</sup> is Na). The results are shown in Table 1.

Table 1

Plasmid	Compound (VIII-a) (mg/l)
None	0
pUC119	0
pSTV28	0
pUbioI	0
pScypA	0
pUcypX	0
pUpksS	0
pSyet0	0
pSyjiB	0.6
pSyrhJ	0

Example 2: Expression of *yjiB* gene in *Bacillus subtilis* as a host cell and confirmation of activity of the protein encoded by said gene

Sense and antisense primers having a combination of nucleotide sequences shown by SEQ ID NOS:17 and 18, SEQ ID NOS:19 and 20, SEQ ID NOS:21 and 22, SEQ ID NOS:23 and 24, SEQ ID NOS:25 and 26, SEQ ID NOS:27 and 28, and SEQ ID NOS:29 and 30, were synthesized with a DNA synthesizer.

Using the chromosomal DNA of *Bacillus subtilis* obtained in Example 1 as a template, PCR was performed with these primers and with TaKaRa LA-PCR™ Kit Ver.2 (produced by TAKARA), Expand™ High-FidelityPCR System (produced by Boehringer Mannheim) or Taq DNA polymerase (produced by Boellinger) using a DNA Thermal Cycler (produced by Perkin-Elmer Japan).

PCR was performed for 30 cycles under the conditions where one cycle consists of the reaction steps of 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 72°C for the DNA fragments of 2kb or less, and 20 seconds at 98°C and 3 minutes at 68°C for the DNA fragments of more than 2kb, and then a reaction was carried out for 7 minutes at 72°C.

Among DNA fragments amplified by PCR, the DNA fragment (containing *bioI* gene) amplified by a combination of primers of SEQ ID NOS:17 and 18 was digested with restriction enzymes SpeI and BamHI, DNA fragment (containing *cypA* gene) amplified by a combination of primers of SEQ ID NOS:19 and 20 was digested with SpeI and BamHI, DNA fragment (containing *cypX* gene) amplified by a combination of primers of SEQ ID NOS:21 and 22 was digested with SpeI and NruI, DNA fragment (containing *pksS* gene) amplified by a combination of primers of SEQ ID NOS:23 and 24 was digested with SpeI and BamHI, DNA fragment (containing *yet0* gene) amplified by a combination of primers of SEQ ID NOS:25 and 26 was digested with SpeI and BamHI, DNA fragment (containing *yjiB* gene) amplified by a combination of primers of SEQ ID NOS:27 and 28 was digested with SpeI and BamHI, DNA fragment (containing *yrhJ* gene) amplified by a combination of primers of SEQ ID NOS:29 and 30 was digested with SpeI and BamHI, respectively.



After digestion, the DNA fragments treated with the restriction enzymes were subjected to agarose gel electrophoresis to obtain the DNA fragments treated with each restriction enzyme.

A vector plasmid pWH1520 (produced by MoBiTec) was digested with restriction enzymes SpeI and BamHI, then subjected to agarose gel electrophoresis to obtain a SpeI-BamHI treated pWH1520 fragment. Similarly, a vector plasmid pWH1520 was digested with restriction enzymes SpeI and NruI, then subjected to agarose gel electrophoresis to obtain a SpeI-NruI pWH1520 fragment.

The thus-obtained SpeI-BamHI treated DNA fragments (amplified by PCR with a combination of primers of SEQ ID NOS:17 and 18, SEQ ID NOS:19 and 20, SEQ ID NOS:23 and 24, SEQ ID NOS:25 and 26, SEQ ID NOS:27 and 28, and SEQ ID NOS:29 and 30) were mixed with the SpeI-BamHI treated pWF1520 fragment; SpeI-NruI treated DNA fragment (amplified by PCR with a combination of primers of SEQ ID NOS:21 and 22) was mixed with SpeI-NruI pWF1520 fragment, respectively. After ethanol precipitation, the obtained DNA precipitates were dissolved in 5  $\mu$ l of distilled water, and a ligation reaction was carried out to obtain each recombinant DNA.

Using the recombinant DNA, *E.coli* (purchased from TOYOBO) DH5  $\alpha$  strain was transformed by a usual method, then plated to a LB agar medium containing 10  $\mu$ g/ml of tetracycline, and cultured for 2 days at 25°C. Cells were recovered from the obtained culture by centrifugation.

A plasmid was isolated from the cells in a usual manner.

The structure of the isolated plasmid was examined by cleaving it with various restriction enzymes and the nucleotide sequences thereof were determined, thereby confirming that the desired DNA fragment was inserted in the plasmid. The plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:17 and 18 to pWH1520 was named as pWbioI; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:19 and 20 to pWH1520 was named as pWcypA; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:21

and 22 to pWH1520 was named as pWcypX; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:23 and 24 to pWH1520 was named as pWpksS; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:25 and 26 to pWH1520 was named as pWyet0; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:27 and 28 to pWH1520 was named as pWyiB; the plasmid obtained by linking the DNA fragment amplified by PCR with a combination of primers of SEQ ID NOS:29 and 30 to pWH1520 was named as pWyrhJ, respectively.

The thus-obtained plasmids and the vector plasmid pWH1520 were introduced in a *Bacillus subtilis* ATCC33712 strain according to the method by S.chang and S.N. cohen [S. chang and S.N. cohen: Mol. Gen. Genet., 168, 111 (1979).]

That is, ATCC33712 strain was inoculated in a thick tube containing 5ml of Pen medium (where 1.75 g of Difco Antibiotic medium No. 3 was dissolved in 100ml of water and sterilized in an autoclave), then cultured with shaking at 37°C overnight. Total cells cultured overnight in 300ml Erlenmeyer flask containing 100ml of Pen medium were then inoculated and cultured with shaking for 3 hours at 37°C to be grown until reaching a metaphase of exponential growth. The culture was centrifuged for 10 minutes at 5000rpm in germ-free conditions to precipitate the cells. After removing the supernatant, the cells were suspended in 4.5ml of SMMP [mixture comprising equal amount of 2 x SMMP (where sucrose 34.2g, maleic acid 0.464g, magnesium chloride·6H<sub>2</sub>O 0.813g were dissolved in water, which was adjusted to pH6.5 with sodium hydroxide, then the final volume of 100ml was sterilized in an autoclave) and 4 x Pen medium (where 7g of Difco Antibiotic medium No. 3 was dissolved in 100ml of water and sterilized in an autoclave)], followed by addition of 0.5ml of lysozyme solution [where 10mg of lysozyme (produced by EIKAGAKU corp.) was dissolved in 0.5ml of SMMP, and sterilized with a millipore filter having a pore size of 0.45μm], and the mixture was slowly shaken for 2 hours at 37°C. After microscopically confirming that not less than 90% cells became protoplast, the

protoplasts were centrifuged for 20 minutes at 3000rpm to be precipitated. The supernatant was removed, and the obtained protoplasts were resuspended in 5 ml of SMMP. The protoplasts were collected by centrifugation for 20 minutes at 3000 rpm, and suspended in 2ml of SMMP to prepare a protoplast suspension of a recipient strain for transformation.

Approximately 1  $\mu$ g of plasmid DNA was dissolved in SMMP, and thoroughly mixed with 0.5ml of protoplast suspension. Immediately after mixing, 1.5ml of 40% polyethylene glycol solution [where 40g of polyethylene glycol 6000 (Nacalai tesque) was dissolved in 2 x SMMP, and water was added thereto to become the volume of 100ml, followed by sterilization in an autoclave] was added thereto and thoroughly mixed. After standing at room temperature for 2 minutes, 5ml of SMMP was added and mixed, and the mixture was centrifuged for 20 minutes at 3000rpm. After removing the supernatant, the precipitated protoplasts were suspended in 1ml of SMMP, then slowly shaken for 3 hours at 30°C. After dilution with SMMP as appropriate, the protoplasts were applied to a DM3 medium [in which 45ml of 80g/L bactoagar (produced by Difco), 50ml of 50g/L casamino acid, 250ml of 338g/L sodium succinate  $\cdot$  6H<sub>2</sub>O (pH7.3), 50ml phosphate buffer (35g/L potassium hydrogen phosphate, 15g/L potassium dihydrogen phosphate), 25ml of 100g/L yeast extract, 10ml of 203g/L magnesium chloride  $\cdot$  6H<sub>2</sub>O, 25ml of 100g/L glucose were respectively sterilized in an autoclave and mixed, then 3.5ml of 20mg/ml bovine serum albumin sterilized with millipore filter having a pore size of 0.45  $\mu$ m was added thereto] wherein the medium containing drugs (in case of tetracycline, it was added to the final volume of 10  $\mu$ g/ml). The protoplasts were cultured for 1 to 2 days at 37°C to obtain the transfected strain.

Thus, *B. subtilis* ATCC33712 strains having each of the above plasmids were obtained.

The obtained transformants and ATCC33712 strain having no plasmid were inoculated respectively in 3ml LB liquid media (wherein 10mg/l tetracycline was added to a plasmid-containing strain), and cultured with shaking for 24 hours at 30°C. 0.25ml of this culture solution was inoculated in a test tube containing a 5ml of TB

medium [Bacto Trypton (produced by Difco) 1.4%, Bacto yeast extract (produced by Difco) 2.4%,  $\text{KH}_2\text{PO}_4$  0.231%, and  $\text{K}_2\text{HPO}_4$  1.251%, adjusted to pH7.4 with 1mol/l sodium hydroxide], and cultured with shaking for 3 hours at 30°C. After 3 hours, 1ml of the culture was transferred to an assist tube No. 60.540S (produced by ASSIST) and 40  $\mu\text{l}$  of 50% sterilized xylose solution was added thereto, followed by culturing with shaking for 3 hours. Then, the compound (VII-a) (wherein R is Na) obtained in Example 1 was added to each test tube to the final concentration of 0.2mg/ml, and the mixture was cultured with shaking for 16 hours at 30°C.

Upon completion of reaction, the reaction solution was adjusted to pH 3.5 with acetic acid. 1ml of ethyl acetate was added to 0.5ml of this reaction solution, and the mixture was shaken for 1 hour. After shaking, the reaction solution was centrifuged for 5 minutes at 3000rpm to be separated into 2 layers, and the upper ethyl acetate layer was recovered, the solvent was removed by a centrifugal evaporator, and the residue was dissolved in 0.5ml of methanol.

Using an aliquot of this methanol solution, HPLC analysis was performed as in Example 1 to detect and quantify compound (VIII-a) (wherein  $\text{R}^1$  is Na). The results are shown in Table 2.

Table 2

Plasmid	Compound (VIII-a) (mg/l)
None	0.5
pWH1520	0.5
pWbioI	0.5
pWcypA	0.5
pWcypX	0.5
pWpksS	0.5
pWyet0	0.5
pWyjiB	24.6
pWyrhJ	0.5

As seen from the results of Example 1 and 2, it is obvious that activity of producing compound (VIII-a) or compound (VIII-b) from compound (VII-a) or compound (VII-b) is encoded by *yjiB* gene.

The DNA fragment amplified by PCR with a combination of primers of SEQ ID NO:27 and 28 above, contained the nucleotide sequence shown by SEQ ID NO:2; and said nucleotide sequence contained a nucleotide sequence encoding a protein having the amino acid sequence shown by SEQ ID NO:1.

Example 3: Expression of *yjiB* gene using *Bacillus megaterium* as a host cell and production of compound (VIII-a)

pWyjiB prepared in Example 2 was introduced into *Bacillus megaterium* (produced by MoBiTec) and *Bacillus* sp. FERM BP-6030 in the same manner as is described for transformation of *Bacillus subtilis* in Example 2.

The obtained transformant and a host cell having no plasmid were cultured and reaction was carried out in the same manner as in Example 2, and the amount of produced compound (VIII-a) was measured. The results are shown in Table 3.

Table 3

Host	Plasmid	Compound (VIII-a) (mg/l)
<i>B.megaterium</i>	none	2.0
(as above)	pWyjiB	27.2
FERM BP-6030	none	4.5
(as above)	pWyjiB	30.3

Example 4: Preparation of the plasmid for expressing the protein which produces compound (VIII-a) in coryne-form bacteria

To allow efficient expression of *yjiB* gene obtained in Example 1 in coryne-form bacteria, DNAs having nucleotide sequences shown by SEQ ID NOS:31,

32, 33, 34, 35, 36, 37, 38 and 39 were synthesized with a DNA synthesizer.

The plasmid pR1109 DNA in which the DNA fragment comprising a promoter sequence p54-6 (GenBank AJ132582) for expression in coryne-form bacteria and having the nucleotide sequence shown by SEQ ID NO:40 was inserted into a Sse83871-BamHI site of a plasmid vector pCS299P (Japanese Patent Application No. 11-110437) , was prepared in a usual manner from *E.coli* NM522 strain transformed with this plasmid.

Using pWyjiB DNA obtained in Example 2 as a template, PCR was performed with DNA primers having nucleotide sequences shown by SEQ ID NOS:31 and 32 and with Taq DNA polymerase (produced by TAKARA) using a DNA Thermal Cycler 480 (produced by Perkin-Elmer Japan).

PCR was performed for 25 cycles in which each cycle consists of reaction steps of 30 seconds at 96°C, 45 seconds at 50°C and 3 minutes at 72°C.

DNA fragment amplified by PCR was digested with SalI and BamHI and subjected to agarose gel electrophoresis, and an approximately 1.2kb DNA fragment was purified in a usual manner to obtain a SalI-BamHI treated DNA fragment.

The above-obtained plasmid pRI109 DNA was digested with restriction enzymes SalI and BamHI and subjected to agarose gel electrophoresis, and an approximately 6 kb DNA fragment was purified in a usual manner to obtain a SalI-BamHI treated pRI109 fragment.

The above-obtained SalI-BamHI treated DNA fragment and SalI-BamHI treated pRI109 fragment were mixed, and ligation reaction was carried out to obtain the recombinant DNA.

Using the recombinant DNA, E.coli DH5  $\alpha$  (purchased from TOYOBO) was transformed by a usual method, then plated to a LB agar medium containing 20  $\mu$ g/ml kanamycin and cultured for 1 day at 30°C to obtain the transformant.

A plasmid was isolated from the transformant in a usual manner. Using the isolated plasmid DNA as a template, and using DNAs having nucleotide sequences shown by SEQ ID NOS:33, 34, 35, 36 and 37 as primers respectively, the nucleotide

sequences of the inserted DNA fragments were determined with a DyeTerminator Cycle Sequencing Kit (produced by Applied Biosystem) and 373A sequencer (produced by Applied Biosystem), then the plasmid in which the nucleotide sequence shown by SEQ ID NO:41 was inserted between SalI and BamHI sites of pRI109 was named pRIyjiB.

The nucleotide sequence shown by SEQ ID NO:41 contained the nucleotide sequence which encoded the protein having the amino acid sequence shown by SEQ ID NO:42.

Using the chromosomal DNA of *Bacillus subtilis* Marburg168 strain (ATCC15563) obtained in Example 1 as a template, PCR was performed with DNA primers having nucleotide sequences shown by SEQ ID NOS:38 and 39, and with LA-Taq DNA polymerase (produced by TAKARA) using a DNA Thermal Cycler 480 (produced by Perkin-Elmer Japan).

PCR was performed for 30 cycles in which each cycle consists of reaction steps of 30 seconds at 96°C, 30 seconds at 55°C and 2 minutes at 72°C, and then a reaction was carried out for 7 minutes at 72°C.

The DNA fragment amplified by PCR was mixed with pT7Blue (produced by TAKARA), and ligation reaction was carried out to obtain the recombinant DNA.

Using the recombinant DNA, *E.coli* DH5  $\alpha$  (purchased from TOYOBO) was transformed by a usual method, then plated to a LB agar medium containing 100  $\mu$ g/ml ampicillin and cultured for 1 day at 30°C to obtain the transformant.

A plasmid was isolated from the transformant by a usual method. The structure of the isolated plasmid was examined by cleaving it with various restriction enzymes, thereby confirming that the desired DNA fragment was inserted in the plasmid, and the plasmid was named as pTSYN2-72.

The pTSYN2-72 DNA was digested with XhoI and BamHI and subjected to agarose gel electrophoresis, and then an approximately 1.2kb DNA fragment was purified by a usual method to obtain a XhoI-BamHI treated DNA fragment.

The plasmid pRI109 DNA was digested with restriction enzymes SalI and BamHI and subjected to agarose gel electrophoresis, and then an approximately 6kb

DNA fragment was purified by a usual method to obtain a Sall-BamHI treated pRI109 fragment.

The above-obtained XhoI-BamHI treated DNA fragment and Sall-BamHI treated pRI109 fragment were mixed, and the ligation reaction was carried out to obtain the recombinant DNA.

Using the recombinant DNA, E.coli DH5  $\alpha$  (purchased from TOYOBO) was transformed by a usual method, then plated to a LB agar medium containing 20  $\mu$ g/ml kanamycin and cultured for 1 day at 30°C to obtain a transformant.

A plasmid was isolated from the transformant by a usual method. Using the isolated plasmid DNA as a template, and using DNAs having nucleotide sequences shown by SEQ ID NOS:33, 34, 35, 36 and 37, the nucleotide sequences of the inserted DNA fragment were determined with a DyeTerminator Cycle Sequencing Kit (produced by Applied Biosystem) and 373A sequencer (produced by Applied Biosystem), and the plasmid in which the nucleotide sequence shown by SEQ ID NO:43 was inserted between Sall-BamHI site of pRI109, was named pSYN2-72.

The nucleotide sequence shown by SEQ ID NO:43 contained the nucleotide sequence which encodes the protein having the amino acid sequence shown by SEQ ID NO:1.

Using pWyjiB DNA obtained in Example 2 as a template, PCR was performed with DNA primers having nucleotide sequences shown by SEQ ID NO:38 and 39, and with Z-Taq DNA polymerase (produced by TAKARA) using a DNA Thermal Cycler 480 (produced by Perkin-Elmer Japan).

PCR was performed for 25 cycles in which each cycle consists of reaction steps of 20 seconds at 98°C, 20 seconds at 55°C and 30 minutes at 72°C.

The DNA fragment amplified by PCR was digested with XhoI and BamHI and subjected to agarose gel electrophoresis, and then an approximately 1.2kb DNA fragment was purified by a usual method to obtain a XhoI-BamHI treated DNA fragment.

The plasmid pRI109 DNA was digested with restriction enzymes Sall and



BamHI and subjected to agarose gel electrophoresis, then an approximately 6kb DNA fragment was purified by a usual method to obtain a SalI-BamHI treated pRI109 fragment.

The above-obtained XhoI-BamHI treated DNA fragment and SalI-BamHI treated pRI109 fragment were mixed, and ligation reaction was carried out to obtain the recombinant DNA.

Using the recombinant DNA, E.coli DH5  $\alpha$  (purchased from TOYOBO) was transformed by a usual method, then plated to a LB agar medium containing 20  $\mu$ g/ml kanamycin and cultured for 1 day at 30°C to obtain the transformant.

A plasmid was isolated from the transformant by a usual method. Using the isolated plasmid DNA as a template, and using DNAs having nucleotide sequences shown by SEQ ID NOS:33, 34, 35, 36 and 37 respectively as primers, the nucleotide sequences of the inserted DNA fragments were determined with a DyeTerminator Cycle Sequencing Kit (produced by Applied Biosystem) and 373A sequencer (produced by Applied Biosystem), and the plasmid in which the nucleotide sequence shown by SEQ ID NO:44 was inserted between SalI-BamHI site of pRI109, was named pSYN2-39.

The nucleotide sequence shown by SEQ ID NO:44 contained the nucleotide sequence which encodes the protein having the amino acid sequence shown by SEQ ID NO:45.

Example 5: Introduction of the plasmid into the *C. glutamicum* ATCC13032 strain and evaluation of activity

ATCC13032 strain was inoculated in a test tube containing 8ml of broth medium [20g/l normal broth medium (produced by Kyokuto Pharmaceutical Industry, Co. Ltd), 5g/l Bacto Yeast Extract (produced by Difco)] and cultured with shaking 30°C overnight. Subsequently, 5ml of cells cultured overnight were inoculated in a 2L Erlenmeyer flask (bearing a baffles) containing 250ml of broth medium and cultured with shaking for 4 hours at 30°C. The obtained culture solution was centrifuged to precipitate the cells. After removing the supernatant, the cells were suspended in 30ml

of ice-cold EPB [250mmol/l Sucrose, 15%(v/v) glycerol], and centrifuged to be precipitated. Similarly, the cells were resuspended in EPB and centrifuged to be separated, and then the cells were suspended in 2ml of EPB. The obtained cell suspension was poured into 0.5ml tubes by 0.1ml each, and was quickly frozen with dry ice to obtain the cell suspension for transformation. The obtained cells were stored at a temperature below -80°C.

0.1ml of the frozen cell suspension for transformation was dissolved on ice, retained for 10 minutes at 43.5°C, and transferred onto ice. After 2 $\mu$ l of aqueous solution containing approximately 2 $\mu$ g pRI109 DNA was added, the cell suspension was transferred to the previously iced *E.coli* GenePulser cuvet (produced by BioRad), and then the DNA was introduced into cells under conditions of 25 $\mu$ F, 200  $\Omega$  and 1.5kV by electroporation using GenePulser (produced by BioRad). Immediately after electroporation, total amount of the cell suspension was moved to a 15ml-test tube containing 1ml of broth medium, and cultured with shaking for 1 hour at 30°C.

The obtained culture solution was centrifuged for 10 minutes at 3,500rpm to precipitate the cells. After removing the supernatant, the cells were suspended with addition of 0.1ml broth medium, then the suspension was applied to a broth agar medium [which was solidified with 2% Difco Agar] containing 20 $\mu$ g/ml kanamycin and cultured for 2 days at 30°C to obtain the transformant.

Thus, *C.glutamicum* ATCC13032 strain having pRI109 was obtained.

As in the above, *C.glutamicum* ATCC13032 strains having each plasmid, pRIyjiB, pSYN2-72, pSYN2-39 were obtained.

The obtained transformants were inoculated in test tubes which contain 3ml of broth media containing 100 $\mu$ g/ml kanamycin, and cultured with shaking for 24 hours at 30°C. The culture (0.2 ml) was inoculated in a test tube containing 2ml of LMC medium [in which separately sterilized Glucose, MgSO<sub>4</sub>, FeSO<sub>4</sub>, MnSO<sub>4</sub> were added to a pre-LMC medium sterilized in a autoclave (NH<sub>4</sub>Cl 1g/l, KH<sub>2</sub>PO<sub>4</sub> 1g/l, K<sub>2</sub>HPO<sub>4</sub> 3g/l, Difco Yeast Extract 0.2g/l, Urea 1g/l, Biotin 0.05mg/l, Thiamin 0.5mg/l, Corn Steep Liquor 10g/l; pH7.2) to the final concentration of 30g/l, 0.1g/l, 2mg/l and 2mg/l,

respectively] wherein the medium contains 100  $\mu$ g/ml kanamycin, and cultured with shaking for 5 hours at 30°C. The compound (VII-a) (wherein R is Na) was added thereto to the final concentration of 300mg/l, and the mixture was reacted with shaking for 16 hours at 30°C.

0.5ml of the reaction solution was moved to a 1.5ml tube, and centrifuged for 2 minutes at 15,000rpm to separate the cells. The obtained supernatant was diluted 5 to 20 times with methanol and centrifuged for 2 minutes at 15,000rpm, and then an aliquot thereof was used for HPLC analysis as in Example 1 to detect and quantify the compound (VIII-a) (wherein R<sup>1</sup> is Na). The concentration of the compound (VIII-a) in the reaction solution calculated based on the quantification result, is shown in Table 4.

Table 4

Plasmid	Compound (VIII-a)(mg/l)
pRI109	0.3
pSYN2-72	30
pRIyjiB	61
pSYN2-39	104

Example 6: Introduction of the plasmid into coryne-form bacteria and evaluation of activity

pRIyjiB DNA obtained in Example 4 was introduced into *C.callunae* ATCC15991, *C.ammoniagenes* ATCC6872 and *B.flavum* ATCC14067 in the same manner as in the transformation of ATCC13032 strain described in Example 5, and transformants were obtained from each strain.

The obtained transformants were respectively inoculated on 3ml of broth media in test tubes containing 100  $\mu$ g/ml kanamycin, and cultured with shaking for 24 hours at 30°C. The culture (0.5ml) was transferred to a test tube containing 5ml TB medium [in which 14g of Bacto Trypton (produced by Difco) and 24g of Bacto Yeast Extract (produced by Difco) were dissolved in 900ml of water and sterilized in an autoclave, to

which 100ml PB [ $\text{KH}_2\text{PO}_4$  23.1g/l,  $\text{K}_2\text{HPO}_4$  125.1g/l] separately sterilized in an autoclave was added] wherein the medium contains 100  $\mu\text{g/ml}$  kanamycin and 10g/l Glucose, and cultured with shaking for 5 hours at 30°C. The culture (1ml) was transferred to an assist tube (produced by ASSIST), and compound (VII-a) (wherein R is Na) was added thereto to the final concentration of 300mg/l, and the mixture was reacted with shaking for 16 hours at 30°C.

Upon completion of reaction, compound (VIII-a) (wherein  $\text{R}^1$  is Na) in the culture was detected and quantified in the method as in Example 2. The concentration of compound (VIII-a) in the culture calculated based on the quantification results, is shown in Table 5.

Table 5

Host Cell	Plasmid	Compound (VIII-a) (mg/l)
<i>C.callunae</i> ATCC15991 (KY3510)	pRIyjiB	22
<i>C.ammoniagenes</i> ATCC6872 (KY3454)	pRIyjiB	12
<i>B.flavum</i> ATCC14067 (KY10122)	pRIyjiB	23

#### Industrial Applicability

The present invention enables efficient production of a DNA encoding a novel hydroxylase and a compound inhibiting hydroxymethylglutaryl CoA (HMG-CoA) reductase and has an action of reducing serum cholesterol.

#### Free Text of Sequence Listing

SEQ ID NO:3    synthetic DNA  
 SEQ ID NO:4    synthetic DNA  
 SEQ ID NO:5    synthetic DNA  
 SEQ ID NO:6    synthetic DNA  
 SEQ ID NO:7    synthetic DNA  
 SEQ ID NO:8    synthetic DNA

SEQ ID NO:9 synthetic DNA  
SEQ ID NO:10 synthetic DNA  
SEQ ID NO:11 synthetic DNA  
SEQ ID NO:12 synthetic DNA  
SEQ ID NO:13 synthetic DNA  
SEQ ID NO:14 synthetic DNA  
SEQ ID NO:15 synthetic DNA  
SEQ ID NO:16 synthetic DNA  
SEQ ID NO:17 synthetic DNA  
SEQ ID NO:18 synthetic DNA  
SEQ ID NO:19 synthetic DNA  
SEQ ID NO:20 synthetic DNA  
SEQ ID NO:21 synthetic DNA  
SEQ ID NO:22 synthetic DNA  
SEQ ID NO:23 synthetic DNA  
SEQ ID NO:24 synthetic DNA  
SEQ ID NO:25 synthetic DNA  
SEQ ID NO:26 synthetic DNA  
SEQ ID NO:27 synthetic DNA  
SEQ ID NO:28 synthetic DNA  
SEQ ID NO:29 synthetic DNA  
SEQ ID NO:30 synthetic DNA  
SEQ ID NO:31 synthetic DNA  
SEQ ID NO:32 synthetic DNA  
SEQ ID NO:33 synthetic DNA  
SEQ ID NO:34 synthetic DNA  
SEQ ID NO:35 synthetic DNA  
SEQ ID NO:36 synthetic DNA  
SEQ ID NO:37 synthetic DNA

SEQ ID NO:38 synthetic DNA

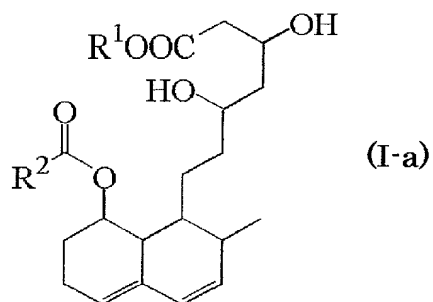
SEQ ID NO:39 synthetic DNA

SEQ ID NO:40 synthetic DNA

## CLAIMS

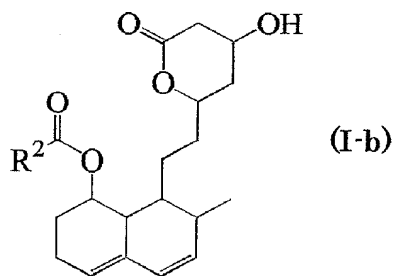
1. A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b),

wherein the compound (I-a) is a compound represented by the formula (I-a):



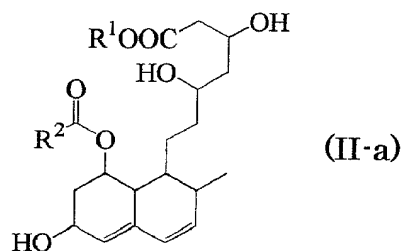
wherein R<sup>1</sup> represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal, and R<sup>2</sup> represents a substituted or unsubstituted alkyl, or a substituted or unsubstituted aryl;

the compound (I-b) is a lactone form of compound (I-a) and is represented by the formula (I-b):



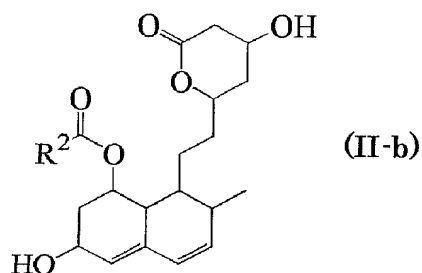
wherein  $R^2$  has the same definition as the above;

the compound (II-a) is a compound represented by the formula (II-a):



wherein  $R^1$  and  $R^2$  have the same definitions as the above; and

the compound (II-b) is a lactone form of compound (II-a) and is represented by the formula (II-b):

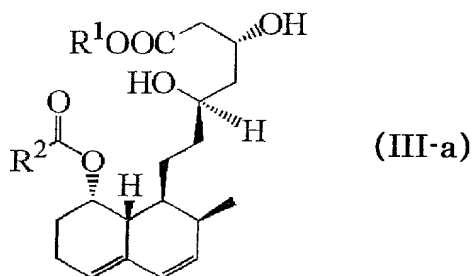


wherein  $R^2$  has the same definition as the above.

2. A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (IV-a) or compound (IV-b) from compound (III-a) or compound (III-b),

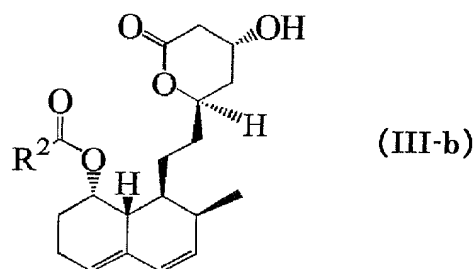
wherein the compound (III-a) is a compound represented by the formula (III-a):





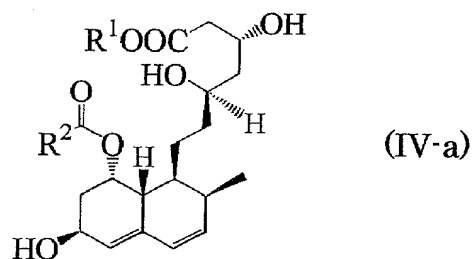
wherein R<sup>1</sup> represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal, and R<sup>2</sup> represents a substituted or unsubstituted alkyl, or a substituted or unsubstituted aryl;

the compound (III-b) is a lactone form of compound (III-a) and is represented by the formula (III-b):



wherein R<sup>2</sup> has the same definition as the above;

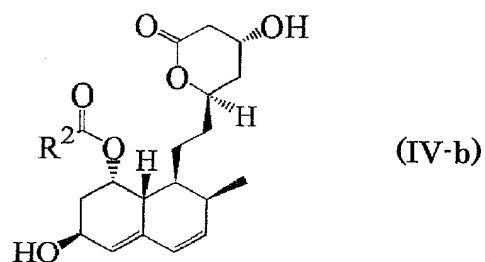
the compound (IV-a) is a compound represented by the formula (IV-a):



wherein R<sup>1</sup> and R<sup>2</sup> have the same definitions as the above; and

the compound (IV-b) is a lactone form of compound (IV-a) and is represented by the

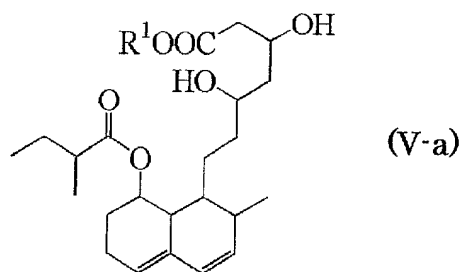
formula (IV-b):



wherein  $R^2$  has the same definition as the above.

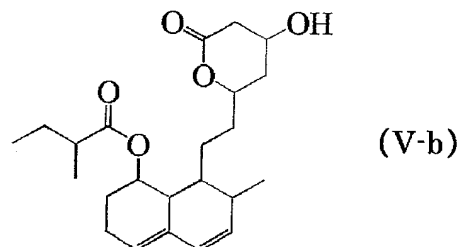
3. A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (VI-a) or compound (VI-b) from compound (V-a) or compound (V-b),

wherein the compound (V-a) is a compound represented by the formula (V-a):

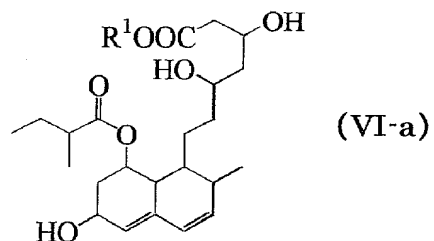


wherein  $R^1$  represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal;

the compound (V-b) is a lactone form of compound (V-a) and is represented by the formula (V-b):

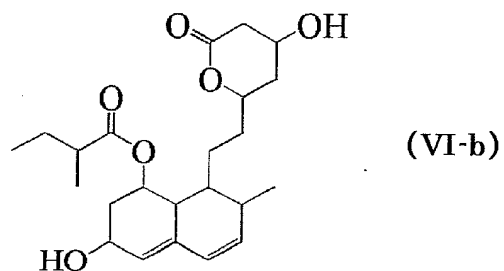


the compound (VI-a) is a compound represented by the formula (VI-a):



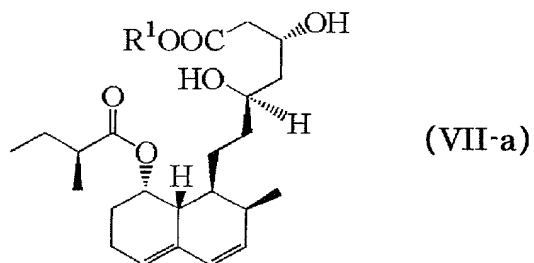
wherein R<sup>1</sup> has the same definition as the above; and

the compound (VI-b) is a lactone form of the compound (VI-a) and is represented by the formula (VI-b):



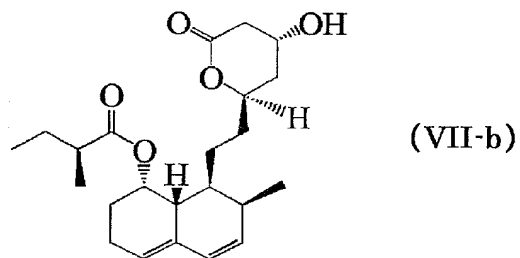
4. A protein which is derived from a microorganism belonging to the genus *Bacillus*, and has an activity of producing compound (VIII-a) or compound (VIII-b) from compound (VII-a) or compound (VII-b),

wherein the compound (VII-a) is a compound represented by the formula (VII-a) :

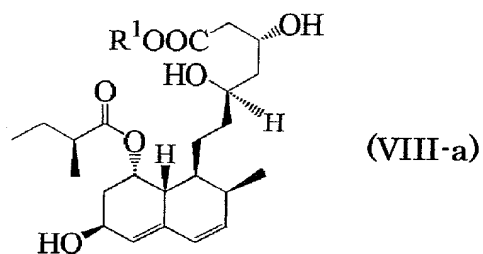


wherein  $R^1$  represents a hydrogen atom, a substituted or unsubstituted alkyl, or an alkali metal;

the compound (VII-b) is a lactone form of compound (VII-a) and is represented by the formula (VII-b):

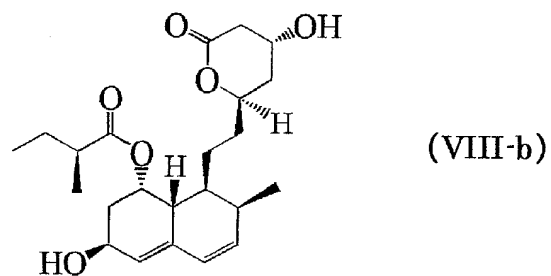


the compound (VIII-a) is a compound represented by the formula (VIII-a):



wherein  $R^1$  has the same definition as the above; and

the compound (VIII-b) is a lactone form of compound (VIII-a) and is represented by the formula (VIII-b):



5. The protein according to any one of claims 1 to 4, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis*, *B. megaterium*, *B. laterosporus*, *B. sphaericus*, *B. pumilus*, *B. stearothermophilus*, *B. cereus*, *B. badius*, *B. brevis*, *B. alvei*, *B. circulans* and *B. macerans*.

6. The protein according to any one of claim 1 to 5, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *B. subtilis* ATCC6051, *B. megaterium* ATCC10778, *B. megaterium* ATCC11562, *B. megaterium* ATCC13402, *B. megaterium* ATCC15177, *B. megaterium* ATCC15450, *B. megaterium* ATCC19213, *B. megaterium* IAM1032, *B. laterosporus* ATCC4517, *B. pumilus* FERM BP-2064, *B. badius* ATCC14574, *B. brevis* NRRL B-8029, *B. alvei* ATCC6344, *B. circulans* NTCT-2610, and *B. macerans* NCIMB-9368.

7. The protein according to any one of claims 1 to 5, wherein the microorganism belonging to the genus *Bacillus* is a microorganism selected from *Bacillus* sp. FERM BP-6029 or *Bacillus* sp. FERM BP-6030.

8. A protein having the amino acid sequence shown by SEQ ID NO: 1.

9. A protein which has an amino acid sequence comprising deletion, substitution or addition of one or more amino acids in the amino acid sequence shown by SEQ ID NO: 1, and has an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b).

10. The protein according to claim 9, wherein the protein has the amino acid sequence shown by SEQ ID NO: 42 or 45.

11. The protein according to claim 9, wherein the compound (I-a) is compound (III-a), the compound (I-b) is compound (III-b), the compound (II-a) is compound (IV-a), and the compound (II-b) is compound (IV-b).
12. The protein according to claim 9, wherein the compound (I-a) is compound (V-a), the compound (I-b) is compound (V-b), the compound (II-a) is compound (VI-a), and the compound (II-b) is compound (VI-b).
13. The protein according to claim 9, wherein the compound (I-a) is compound (VII-a), the compound (I-b) is compound (VII-b), the compound (II-a) is compound (VIII-a), and the compound (II-b) is compound (VIII-b).
14. An isolated DNA having the nucleotide sequence shown by SEQ ID NO: 2.
15. An isolated DNA which hybridizes with the DNA according to claim 14 under stringent conditions, and encodes a protein having an activity of producing compound (II-a) or compound (II-b) from compound (I-a) or compound (I-b).
16. The DNA according to claim 15, wherein the DNA has a nucleotide sequence selected from the group consisting of the nucleotide sequences shown by SEQ ID NOS: 41, 43 and 44.
17. An isolated DNA encoding the protein according to any one of claims 1 to 12.
18. The DNA according to claim 15, wherein the compound (I-a) is compound (III-a), the compound (I-b) is compound (III-b), the compound (II-a) is compound (IV-a), and the compound (II-b) is compound (IV-b).
19. The DNA according to claim 15, wherein the compound (I-a) is compound (V-a),

the compound (I-b) is compound (V-b), the compound (II-a) is compound (VI-a), and the compound (II-b) is compound (VI-b).

20. The DNA according to claim 15, wherein the compound (I-a) is compound (VII-a), the compound (I-b) is compound (VII-b), the compound (II-a) is compound (VIII-a), and the compound (II-b) is compound (VIII-b).

21. A recombinant DNA vector comprising the DNA according to any one of claims 14 to 20.

22. A transformant obtained by introducing the recombinant DNA vector according to claim 21 into a host cell.

23. The transformant according to claim 22, wherein the transformant belongs to a microorganism selected from the genera *Escherichia*, *Bacillus*, *Corynebacterium*, and *Streptomyces*.

24. The transformant according to claim 22 or 23, wherein the transformant belongs to microorganism selected from *Escherichia coli*, *Bacillus subtilis*, *Bacillus megaterium*, *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Corynebacterium callunae* and *Streptomyces lividans*.

25. A process for producing compound (II-a) or compound (II-b), wherein the transformant according to any one of claims 22 to 24, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:  
allowing compound (I-a) or compound (I-b) to exist in an aqueous medium;  
allowing compound (II-a) or compound (II-b) to be produced and accumulated in said aqueous medium; and  
collecting compound (II-a) or compound (II-b) from said aqueous medium.

26. A process for producing compound (IV-a) or compound (IV-b), wherein the transformant according to any one of claims 22 to 24, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:  
allowing compound (III-a) or compound (III-b) to exist in an aqueous medium;  
allowing compound (IV-a) or compound (IV-b) to be produced and accumulated in said aqueous medium; and  
collecting compound (IV-a) or compound (IV-b) from said aqueous medium.

27. A process for producing compound (VI-a) or compound (VI-b), wherein the transformant according to any one of claims 22 to 24, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:  
allowing compound (V-a) or compound (V-b) to exist in an aqueous medium;  
allowing compound (VI-a) or compound (VI-b) to be produced and accumulated in said aqueous medium; and  
collecting compound (VI-a) or compound (VI-b) from said aqueous medium.

28. A process for producing compound (VIII-a) or compound (VIII-b), wherein the transformant according to any one of claims 22 to 24, a culture of the transformant, or a treated product of the culture is used as an enzyme source, and the process comprises:  
allowing compound (VII-a) or compound (VII-b) to exist in an aqueous medium;  
allowing compound (VIII-a) or compound (VIII-b) to be produced and accumulated in said aqueous medium; and  
collecting compound (VIII-a) or compound (VIII-b) from said aqueous medium.

29. The process according to claim 25, wherein the compound (II-b) is the compound (II-b) obtained by forming a lacton from compound (II-a).

30. The process according to claim 25, wherein the compound (II-a) is the compound



(II-a) obtained by opening the lactone ring of compound (II-b).

31. The process according to claim 26, wherein the compound (IV-b) is the compound (IV-b) obtained by forming a lacton from compound (IV-a).

32. The process according to claim 26, wherein the compound (IV-a) is the compound (IV-a) obtained by opening the lactone ring of compound (IV-b).

33. The process according to claim 27, wherein the compound (VI-b) is the compound (VI-b) obtained by forming a lacton from compound (VI-a).

34. The process according to claim 27, wherein the compound (VI-a) is the compound (VI-a) obtained by opening the lactone ring of compound (VI-b).

35. The process according to claim 28, wherein the compound (VIII-b) is the compound (VIII-b) obtained by forming a lacton from compound (VIII-a).

36. The process according to claim 28, wherein the compound (VIII-a) is the compound (VIII-a) obtained by opening the lactone ring of compound (VIII-b).

37. The process according to any one of claims 25 to 28, wherein the treated product of the culture of the transformant is a treated product selected from cultured cells; treated products such as dried cells, freeze-dried cells, cells treated with a surfactant, cells treated with an enzyme, cells treated by ultrasonication, cells treated by mechanical milling, cells treated by solvent; a protein fraction of a cell; and an immobilized products of cells or treated cells.

38. A process for producing a protein, which comprises culturing the transformant according to any one of claims 22 to 24 in a medium; producing and accumulating the

protein according to any one of claims 1 to 12 in the culture; and collecting said protein from said culture.

39. An oligonucleotide corresponding to a sequence consisting of 5 to 60 continuous nucleotides in a nucleotide sequence selected from the group consisting of the nucleotide sequences shown by SEQ ID NOS: 2, 41, 43 and 44; or an oligonucleotide corresponding to a complementary sequence to said oligonucleotide.

## Declaration and Power of Attorney for Utility or Design Patent Application

## 特許出願宣言書

## Japanese Language Declaration

私は、下欄に氏名を記載した発明者として、以下のとおり宣言する：

私の住所、郵便の宛先および国籍は、下欄に氏名に続いて記載したとおりであり、

名称の発明に関し、請求の範囲に記載した特許を求める主題の本来の、最初にして唯一の発明者である（一人の氏名のみが下欄に記載されている場合）か、もしくは本来の、最初にして共同の発明者である（複数の氏名が下欄に記載されている場合）と信じ、

上記発明の明細書（下記の欄で x 印がついていない場合は、本書に添付）は、

☒ 年 月 日に提出され、米国出願番号

とし、（該当する場合）

年 月 日に訂正されました。又は、

特許協定条約国際出願番号 とし、

（該当する場合） 年 月 日に訂正されました。

私は、前記のとおり補正した請求の範囲を含む前記明細書の内容を検討し、理解したことを陳述する。

私は、連邦規則法典第 37 編第 1 条 56 項に定義されているとおり、特許資格の有無について重要な情報を開示すべき義務があることを認めます。

私は、合衆国法典第 35 部第 119 条 (a-d) 項又は第 365 条 (b) 項に基づく、下記の外国特許出願又は発明者証出願、或いは第 365 条 (a) 項に基づく、少なくとも米国以外の 1 カ国を指名した PCT 国際出願の外国優先権を主張し、更に優先権の主張に係わる基礎出願の出願日前の出願日を有する外国特許出願、又は発明者証出願、或いは PCT 国際出願を以下に“なし”の箱に印をつけることにより明記する：

## Prior foreign applications

先の外国出願

<u>11-21707</u>	<u>Japan</u>	<u>29/Jan/99</u>
(Number)	(Country)	(Day/Month/Year Filed)
(番号)	(国名)	(出願の年月日)

<u>                    </u>	<u>                    </u>	<u>                    </u>
(Number)	(Country)	(Day/Month/Year Filed)
(番号)	(国名)	(出願の年月日)

☐ その他の外国特許出願番号は別紙の追補優先権欄にて記載する。

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name:

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**Process For Producing HMG-CoA Reductase Inhibitors**

the specification of which is attached hereto unless the following box is checked:

☒ was filed on 28/Jan/00 as United States Application Number 09/869,334 and was amended on 26/Jul/01 (if applicable) or,

PCT International Application Number PCT/JP00/00472 and was amended on                      (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority under Title 35, United States Code §119(a-d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States, listed below. I have also identified below, by checking the "No" box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed:

Priority claimed  
優先権の主張

☒ ☐  
Yes No  
あり なし

☐ ☐  
Yes No  
あり なし

☐ Additional foreign application numbers are listed a supplemental priority sheet attached hereto.

# Japanese Language Utility or Design Patent Application Declaration

私は、合衆国法典第 35 部第 119 条(e)項に基づく、下記の合衆国仮特許出願の利益を主張する。

(Application No.)  
(出願番号)

(Application No.)  
(出願番号)

(Application No.)  
(出願番号)

☐ その他の合衆国仮特許出願番号は別紙の追補優先権欄にて記載する。

私は、合衆国法典第 35 部第 120 条に基づく下記の合衆国特許出願、又は第 365 条(c)項に基づく合衆国を指名した PCT 国際出願の利益を主張し、本願の請求の範囲各項に記載の主題が合衆国法典第 35 部第 112 条第 1 項規定の態様で、先の合衆国特許出願又は PCT 国際出願に開示されていない限度において、先の出願の出願日と本願の国内出願日又は PCT 国際出願日の間に有効となった連邦規則法典第 37 部第 1 章第 56 条に記載の特許要件に所要の情報を開示すべき義務を有することを認める。

(Application No.)  
(出願番号)

(Day/Month/Year Filed)  
(出願の年月日)

(Application No.)  
(出願番号)

(Day/Month/Year Filed)  
(出願の年月日)

☐ その他の合衆国又は国際特許出願番号は別紙の追補優先権欄にて記載する。

私は、ここに自己の知識に基づいて行った陳述が全て真実であり、自己の有する情報および信ずるところに従って行った陳述が真実であると信じ、さらに故意に虚偽の陳述等を行った場合、合衆国法典第 18 部第 1001 条により、罰金もしくは禁に処せられるか、またはこれらの刑が併科され、またかかる故意による虚偽による陳述が本願ないし本願に対して付与される特許の有効性を損なうことがあることを認識して、以上の陳述を行ったことを宣言する。

私、下記署名者は、ここに記載の米国弁護士または代理人に本出願に関し特許商標庁にて取られるいかなる行為に関して、同米国弁護士又は代理人が私に直接連絡なしに私の外国弁護士或いは法人代表者からの指示を受け取り、それに従うようここに委任する。この指示を出す者が変更の場合には、ここに記載の米国弁護士又は代理人にその旨通知される。

I hereby claim the benefit under Title 35, United States Code §119 (e) of any United States provisional application(s) listed below.

(Day/Month/Year Filed)  
(出願の年月日)

(Day/Month/Year Filed)  
(出願の年月日)

(Day/Month/Year Filed)  
(出願の年月日)

☐ Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or §365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(現況) (Status)  
(特許済み、係属中 放棄済み) (patented, pending, abandoned)

(現況) (Status)  
(特許済み、係属中 放棄済み) (patented, pending, abandoned)

☐ Additional U.S. or international application numbers are listed on a supplemental priority sheet attached hereto.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from either his foreign patent agent or corporate representative, if any, as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

# Japanese Language Utility or Design Patent Application Declaration

委任状： 私は、下記発明者として、下記に明記された顧客番号を伴う以下の弁護士又は、代理人をここに選任し、本順の手続きを遂行すること並びにこれに関する一切の行為を特許商標庁に対して行うことを委任する。そして全ての通信はこの顧客番号宛に發送される。

顧客番号 7055

現在委任された弁護士は下記の通りである。

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Bruce H. Bernstein	Reg. No. 29,027
James L. Rowland	Reg. No. 32,674
Arnold Turk	Reg. No. 33,094

POWER OF ATTORNEY: As a named inventor, I hereby appoint the attorney(s) and/or agent(s) associated with the Customer Number provided below to prosecute this application and transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

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国籍	Citizenship Japan
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同第二共同発明者の署名	Second inventor's signature <i>Yoshiyuki Yonetani</i> September 13, 2001
住所	Residence Machida-shi, Tokyo, Japan JPX
国籍	Citizenship Japan
郵便の宛先	Post Office Address c/o Tokyo Research Laboratories Kyowa Hakko Kogyo Co., Ltd., 6-6, Asahi-machi 3-chome, Machida-shi, Tokyo 194-8533, Japan

(第三またはそれ以降の共同発明者に対しても同様な情報および署名を提供すること。)

(Supply similar information and signature for third and subsequent joint inventors.)

# Japanese Language Utility or Design Patent Application Declaration

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共同発明者の署名	日付	Fourth Inventor's signature <u>Shinichi Hashimoto</u>	Date September 13, 2001
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国籍		Citizenship Japan	
郵便の宛先		Post Office Address c/o Tokyo Research Laboratories Kyowa Hakko Kogyo Co., Ltd., 6-6, Asahi-machi 3-chome, Machida-shi, Tokyo 194-8533, Japan	
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共同発明者の署名	日付	Fifth Inventor's signature <u>Akio Ozaki</u>	Date September 13, 2001
住所		Residence <u>Hofu-shi, Yamaguchi, Japan</u>	JPX
国籍		Citizenship Japan	
郵便の宛先		Post Office Address c/o Tokyo Research Laboratories Kyowa Hakko Kogyo Co., Ltd., 1-1, Kyowa-cho, Hofu-shi, Yamaguchi 747-8522, Japan	
第六の共同発明者の氏名 (該当する場合)		Full name of sixth joint inventor, if any	
共同発明者の署名	日付	Sixth Inventor's signature	Date
住所		Residence	
国籍		Citizenship	
郵便の宛先		Post Office Address	

(それ以降の共同発明者に対しても同様な情報および署名を提供すること。)

(Supply similar information and signature for subsequent joint inventors.)

## SEQUENCE LISTING

&lt;110&gt; KYOWA HAKKO KOGYO CO., LTD

&lt;120&gt; A Process for producing HMG-CoA Reductase inhibitor

&lt;130&gt; H11-0011T4

&lt;160&gt; 45

&lt;170&gt; PatentIn Ver. 2.0

&lt;210&gt; 1

&lt;211&gt; 396

&lt;212&gt; PRT

&lt;213&gt; Bacillus subtilis

&lt;400&gt; 1

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Asp Ile Ile Ser Ile Leu Val Glu Ala Glu Glu Thr Gly Glu Lys Leu

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Glu Thr Pro Gly Val Tyr Glu Glu Leu Arg Ser His Pro Glu Leu Met

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Pro Gln Ala Val Glu Glu Ala Leu Arg Phe Arg Ala Pro Ala Pro Val

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Leu Arg Arg Ile Ala Lys Arg Asp Thr Glu Ile Gly Gly His Leu Ile

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Lys Glu Gly Asp Met Val Leu Ala Phe Val Ala Ser Ala Asn Arg Asp

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Glu Ala Lys Phe Asp Arg Pro His Met Phe Asp Ile Arg Arg His Pro

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&lt;210&gt; 2

&lt;211&gt; 1191

&lt;212&gt; DNA

&lt;213&gt; Bacillus subtilis

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&lt;221&gt; CDS

&lt;222&gt; (1)..(1191)

&lt;400&gt; 2

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Met Arg Lys Asp Ala Pro Val Ser Phe Asp Glu Glu Asn Gln Val Trp

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agc gtt ttt ctt tat gat gat gtc aaa aaa gtt gtt ggg gat aaa gag 192

Ser Val Phe Leu Tyr Asp Asp Val Lys Lys Val Val Gly Asp Lys Glu

50

55

60

ttg ttt tcc agt tgc atg ccg cag cag aca agc tct att gga aat tcc 240

Leu Phe Ser Ser Cys Met Pro Gln Gln Thr Ser Ser Ile Gly Asn Ser

65

70

75

80

atc att aac atg gac ccg ccg aag cat aca aaa atc cgt tca gtc gtg 288

Ile Ile Asn Met Asp Pro Pro Lys His Thr Lys Ile Arg Ser Val Val

85

90

95

aac aaa gcc ttt act ccg cgc gtg atg aag caa tgg gaa ccg aga att 336

Asn Lys Ala Phe Thr Pro Arg Val Met Lys Gln Trp Glu Pro Arg Ile

100

105

110

caa gaa atc aca gat gaa ctg att caa aaa ttt cag ggg cgc agt gag 384

Gln Glu Ile Thr Asp Glu Leu Ile Gln Lys Phe Gln Gly Arg Ser Glu

115

120

125

ttt gac ctt gtt cac gat ttt tca tac ccg ctt ccg gtt att gtg ata 432

Phe Asp Leu Val His Asp Phe Ser Tyr Pro Leu Pro Val Ile Val Ile

130

135

140

tct gag ctg ctg gga gtg cct tca gcg cat atg gaa cag ttt aaa gca 480

Ser Glu Leu Leu Gly Val Pro Ser Ala His Met Glu Gln Phe Lys Ala

145

150

155

160

tgg tct gat ctt ctg gtc agt aca ccg aag gat aaa agt gaa gaa gct 528

Trp Ser Asp Leu Leu Val Ser Thr Pro Lys Asp Lys Ser Glu Glu Ala

165

170

175

gaa aaa gcc ttt ttg gaa gaa cga gat aag tgt gag gaa gaa ctg gcc 576

Glu Lys Ala Phe Leu Glu Glu Arg Asp Lys Cys Glu Glu Glu Leu Ala

180

185

190

gcg ttt ttt gcc ggc atc ata gaa gaa aag cga aac aaa ccg gaa cag 624

Ala Phe Phe Ala Gly Ile Ile Glu Glu Lys Arg Asn Lys Pro Glu Gln

195

200

205

gat att att tct att tta gtg gaa gcg gaa gaa aca ggc gag aag ctg 672  
Asp Ile Ile Ser Ile Leu Val Glu Ala Glu Glu Thr Gly Glu Lys Leu  
210 215 220

tcc ggt gaa gag ctg att ccg ttt tgc acg ctg ctg ctg gtg gcc gga 720  
Ser Gly Glu Glu Leu Ile Pro Phe Cys Thr Leu Leu Leu Val Ala Gly  
225 230 235 240

aat gaa acc act aca aac ctg att tca aat gcg atg tac agc ata tta 768  
Asn Glu Thr Thr Thr Asn Leu Ile Ser Asn Ala Met Tyr Ser Ile Leu  
245 250 255

gaa acg cca ggc gtt tac gag gaa ctg cgc agc cat cct gaa ctg atg 816  
Glu Thr Pro Gly Val Tyr Glu Glu Leu Arg Ser His Pro Glu Leu Met  
260 265 270

cct cag gca gtg gag gaa gcc ttg cgt ttc aga gcg ccg gcc ccg gtt 864  
Pro Gln Ala Val Glu Glu Ala Leu Arg Phe Arg Ala Pro Ala Pro Val  
275 280 285

ttg agg cgc att gcc aag cgg gat acg gag atc ggg ggg cac ctg att 912  
Leu Arg Arg Ile Ala Lys Arg Asp Thr Glu Ile Gly Gly His Leu Ile  
290 295 300

aaa gaa ggt gat atg gtt ttg gcg ttt gtg gca tcg gca aat cgt gat 960

Lys Glu Gly Asp Met Val Leu Ala Phe Val Ala Ser Ala Asn Arg Asp

305 310 315 320

gaa gca aag ttt gac aga ccg cac atg ttt gat atc cgc cgc cat ccc 1008

Glu Ala Lys Phe Asp Arg Pro His Met Phe Asp Ile Arg Arg His Pro

325 330 335

aat ccg cat att gcg ttt ggc cac ggc atc cat ttt tgc ctt ggg gcc 1056

Asn Pro His Ile Ala Phe Gly His Gly Ile His Phe Cys Leu Gly Ala

340 345 350

ccg ctt gcc cgt ctt gaa gca aat atc gcg tta acg tct ttg att tct 1104

Pro Leu Ala Arg Leu Glu Ala Asn Ile Ala Leu Thr Ser Leu Ile Ser

355 360 365

gct ttt cct cat atg gag tgc gtc agt atc act ccg att gaa aac agt 1152

Ala Phe Pro His Met Glu Cys Val Ser Ile Thr Pro Ile Glu Asn Ser

370 375 380

gtg ata tac gga tta aag agc ttc cgt gtg aaa atg taa 1191

Val Ile Tyr Gly Leu Lys Ser Phe Arg Val Lys Met

385 390 395

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39

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<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 4

gtgggatccg tcgaccactt ttttcacgat gttcactccc c

41

<210> 5

<211> 39

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<213> Artificial Sequence

<220>

<223> Synthetic DNA

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<210> 6

<211> 39

<212> DNA

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<220>

<223> Synthetic DNA

<400> 6

tcaggatccc ccgggtgagc ggcaaatacca cccaccctg

39

<210> 7

<211> 37

<212> DNA

<213> Artificial Sequence

<220>



<223> Synthetic DNA

<400> 7

taagcgcgcc ccgggttaat tggatgggcg aaagctc

37

<210> 8

<211> 39

<212> DNA

<213> Artificial Sequence

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<223> Synthetic DNA

<400> 8

atcgcgcgcg tcgacgatag cggcagaaaa ttggcggca

39

<210> 9

<211> 38

<212> DNA

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<220>

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agcggatccg aattcgctgg aatcaaaagt cggccaga

38

<210> 10

<211> 38

<212> DNA

<213> Artificial Sequence

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<223> Synthetic DNA

<400> 10

tcaggatccg tcgactgaga aaacacaaaac gccccctc

38

<210> 11

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

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<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

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42

<210> 13

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 13

tctggatcct ctagaagaga acacaaagag tacgaatgc

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<210> 14

<211> 41

<212> DNA

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<400> 14

aaagateccc ccgggtttac cagccagcgc aacaaagtea t

41

<210> 15

<211> 39

<212> DNA

<213> Artificial Sequence

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<223> Synthetic DNA

<400> 15

cctgaattct ctagaaggct ttcaccacgt attttgctg

39

<210> 16

<211> 41

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tctgaattcc ccgggagaac aaaatgccaa aagcctgagtc

41

<210> 17

<211> 34

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<213> Artificial Sequence

<220>

<223> Synthetic DNA

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aatactagta caattgcatc gtcaactgca tctt

34

<210> 18

<211> 41

<212> DNA

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<220>

<223> Synthetic DNA

<400> 18

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41

<210> 19

<211> 34

<212> DNA

<213> Artificial Sequence

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<400> 19

gaaactagtt cttcaaaaga aaaaaagagt gtaa

34

<210> 20

<211> 39

<212> DNA

<213> Artificial Sequence

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<223> Synthetic DNA

<400> 20

tcaggatccc ccgggtgagc ggcaaatacca cccaccctg

39

<210> 21

<211> 34

<212> DNA

<213> Artificial Sequence

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<223> Synthetic DNA

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taaactagta gccaatcgat taaattgttt agtg

34

<210> 22

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 22

ggaggtacct tatgccccgt caaacgcaac gaga

34

<210> 23

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 23

aggactagtc aaatggaaaa attgatgttt catc

34

<210> 24

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

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<400> 24

tcaggatccg tcgactgaga aaacacaaaac gccccctc

38



<210> 25

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 25

ggtactagta aggaaacaag cccgattcct cagc

34

<210> 26

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 26

gccggatcca gatctggcat cacacaacaa taaatacacc gc

42

<210> 27

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

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<400> 27

ttggatccac tagtaatgtg ttaaaccgcc ggcaagcc

38

<210> 28

<211>

<211> 41

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<213> Artificial Sequence

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<223> Synthetic DNA

<400> 28

aaaggatccc ccgggtttac cagccagcgc aacaaagtca t

41

<210> 29

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 29

atgactagta aacaggcaag cgcaatacct cagc

34

<210> 30

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 30

tttggtaacct tacattcctg tccaaacgtc tttc

34

<210> 31

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 31

agcggtcgac aatgaatgtg ttaaaccgc

29

<210> 32

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 32

acgggatcc ttacattttc acacggaag

29

<210> 33

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 33

cgccagggtt ttcccagtca cgac

24

<210> 34

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

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cgcaatatgc ggattggg

18

<210> 35

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 35

tttccggcca ccagcagc

18

<210> 36

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 36

taaccggaag cgggtatg

18

<210> 37

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 37

aaggaaacag gcgcatcc

18

<210> 38

<211> 67

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 38

tcgcctcgag tcgaggaggt cgactaatat gaacgttctg aaccgccgtc aagccttgca 60

gcgagcg

67

<210> 39

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 39

tcgcggatcc ttacattttc acacggaa

28

<210> 40

<211> 715

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 40

cctgcaggtc atcacecgag caggcgaccc gaacgttcgg aggctcctcg ctgtccattc 60

gtccccctgg cgcggtatga accgccgcct catagtgcag ttgatcctg acgagcccag 120

catgtctgcg cccaccttcg cggaacctga ccagggtcgg ctagcgggcg gccggaaggt 180

gaatgctagg catgatctaa ccctcgggtct ctggcgctgc gactgcgaaa tttcgcgagg 240

gtttccgaga aggtgattgc gcttcgcaga tctcgtggac ggcttggttg acgcctccg 300

cccattgggt gatggtggca ccatttggct gttgactcct ggtgcaggaa aacgtggaac 360

tattgtccca ggtgaaatth ccgaatccgc acaattggca ggcctcgtcc agaccaccgc 420

agagcgtctc ggtgattggc agggcagctg cttggtcgcg cgcggcgcca tgaagaagta 480



agaattagcc gaaaacacct tccagccagg cgatttgctt aagttagaag gtgtggctag 540

tattctaaga gtgtcatga ggaagcggaa agcttttaag agagcatgat gcggctttag 600

ctcagctgga agagcaactg gtttacaccc agtaggtcgg gggttcgate cagctgtgaa 660

caattgcact ttggatctaa ttaagggatt agtcgactat ggatccccgg gtacc 715

<210> 41

<211> 1204

<212> DNA

<213> Bacillus subtilis

<220>

<221> CDS

<222> (8)..(1195)

<400> 41

gtcgaca atg aat gtg tta aac cgc egg caa gcc ttg cag cga gcg ctg 49

Met Asn Val Leu Asn Arg Arg Gln Ala Leu Gln Arg Ala Leu

1

5

10

ctc aat ggg aaa aac aaa cag gat gcg tat cat cgc ttt cca tgg tat 97

Leu Asn Gly Lys Asn Lys Gln Asp Ala Tyr His Pro Phe Pro Trp Tyr

15

20

25

30

gaa tcg atg aga aag gat gcg cct gtt tcc ttt gat gaa gaa aac caa 145  
 Glu Ser Met Arg Lys Asp Ala Pro Val Ser Phe Asp Glu Glu Asn Gln  
                   35                                  40                                  45

gtg tgg agc gtt ttt ctt tat gat gat gtc aaa aaa gtt gtt ggg gat 193  
 Val Trp Ser Val Phe Leu Tyr Asp Asp Val Lys Lys Val Val Gly Asp  
                   50                                  55                                  60

aaa gag ttg ttt tcc agt tgc atg ccg cag cag aca agc tct att gga 241  
 Lys Glu Leu Phe Ser Ser Cys Met Pro Gln Gln Thr Ser Ser Ile Gly  
                   65                                  70                                  75

aat tcc atc att aac atg gac ccg ccg aag cat aca aaa atc cgt tca 289  
 Asn Ser Ile Ile Asn Met Asp Pro Pro Lys His Thr Lys Ile Arg Ser  
                   80                                  85                                  90

gtc gtg aac aaa gcc ttt act ccg cgc gcg atg aag caa tgg gaa ccg 337  
 Val Val Asn Lys Ala Phe Thr Pro Arg Ala Met Lys Gln Trp Glu Pro  
                   95                                  100                                  105                                  110

aga att caa gaa atc aca gat gaa ctg att caa aaa ttt cag ggg cgc 385  
 Arg Ile Gln Glu Ile Thr Asp Glu Leu Ile Gln Lys Phe Gln Gly Arg  
                   115                                  120                                  125

agt gag ttt gac ctt gtt cac gat ttt tca tac ccg ctt ccg gtt att 433  
 Ser Glu Phe Asp Leu Val His Asp Phe Ser Tyr Pro Leu Pro Val Ile  
                   130                                  135                                  140

gtg ata tct gag ctg ctg gga gtg cct tca gcg cat atg gaa cag ttt 481

Val Ile Ser Glu Leu Leu Gly Val Pro Ser Ala His Met Glu Gln Phe

145

150

155

aaa gca tgg tct gat ctt ctg gtc agt aca ccg aag gat aaa agt gaa 529

Lys Ala Trp Ser Asp Leu Leu Val Ser Thr Pro Lys Asp Lys Ser Glu

160

165

170

gaa gct gaa aaa gcc ttt ttg gaa gaa cga gat aag tgt gag gaa gaa 577

Glu Ala Glu Lys Ala Phe Leu Glu Glu Arg Asp Lys Cys Glu Glu Glu

175

180

185

190

ctg gcc gcg ttt ttt gcc ggc atc ata gaa gaa aag cga aac aaa ccg 625

Leu Ala Ala Phe Phe Ala Gly Ile Ile Glu Glu Lys Arg Asn Lys Pro

195

200

205

gaa cag gat att att tct att tta gtg gaa gcg gaa gaa aca ggc gag 673

Glu Gln Asp Ile Ile Ser Ile Leu Val Glu Ala Glu Glu Thr Gly Glu

210

215

220

aag ctg tcc ggt gaa gag ctg att ccg ttg tgc acg ctg ctg ctg gtg 721

Lys Leu Ser Gly Glu Glu Leu Ile Pro Leu Cys Thr Leu Leu Leu Val

225

230

235

gcc gga aat gaa acc act aca aac ctg att tca aat gcg atg tac agc 769

Ala Gly Asn Glu Thr Thr Thr Asn Leu Ile Ser Asn Ala Met Tyr Ser

240

245

250

ata tta gaa acg cca ggc gtt tac gag gaa ctg cgc agc cat cct gaa 817

Ile Leu Glu Thr Pro Gly Val Tyr Glu Glu Leu Arg Ser His Pro Glu

255 260 265 270

ctg atg cct cag gca gtg gag gaa gcc ttg cgt ttc aga gcg ccg gcc 865

Leu Met Pro Gln Ala Val Glu Glu Ala Leu Arg Phe Arg Ala Pro Ala

275 280 285

ccg gtt ttg agg cgc att gcc aag cgg gat acg gag atc ggg ggg cac 913

Pro Val Leu Arg Arg Ile Ala Lys Arg Asp Thr Glu Ile Gly Gly His

290 295 300

ctg att aaa gaa ggt gat atg gtt ttg gcg ttt gtg gca tcg gca aat 961

Leu Ile Lys Glu Gly Asp Met Val Leu Ala Phe Val Ala Ser Ala Asn

305 310 315

cgt gat gaa gca aag ttt gac aga ccg cac atg ttt gat atc cgc cgc 1009

Arg Asp Glu Ala Lys Phe Asp Arg Pro His Met Phe Asp Ile Arg Arg

320 325 330

cat ccc aat ccg cat att gcg ttt ggc cac ggc atc cat ttt tgc ctt 1057

His Pro Asn Pro His Ile Ala Phe Gly His Gly Ile His Phe Cys Leu

335 340 345 350

ggg gcc ccg ctt gcc cgt ctt gaa gca aat atc gcg tta acg tct ttg 1105

Gly Ala Pro Leu Ala Arg Leu Glu Ala Asn Ile Ala Leu Thr Ser Leu

355 360 365

att tct gct ttt cct cat atg gag tgc gtc agt atc act ccg att gaa 1153

Ile Ser Ala Phe Pro His Met Glu Cys Val Ser Ile Thr Pro Ile Glu

370

375

380

aac agt gtg ata tac gga tta aag agc ttc cgt gtg aaa atg taaggatcc 1204

Asn Ser Val Ile Tyr Gly Leu Lys Ser Phe Arg Val Lys Met

385

390

395

&lt;210&gt; 42

&lt;211&gt; 396

&lt;212&gt; PRT

&lt;213&gt; Bacillus subtilis

&lt;400&gt; 42

Met Asn Val Leu Asn Arg Arg Gln Ala Leu Gln Arg Ala Leu Leu Asn

1

5

10

15

Gly Lys Asn Lys Gln Asp Ala Tyr His Pro Phe Pro Trp Tyr Glu Ser

20

25

30

Met Arg Lys Asp Ala Pro Val Ser Phe Asp Glu Glu Asn Gln Val Trp

35

40

45

Ser Val Phe Leu Tyr Asp Asp Val Lys Lys Val Val Gly Asp Lys Glu

50

55

60

Leu Phe Ser Ser Cys Met Pro Gln Gln Thr Ser Ser Ile Gly Asn Ser

65

70

75

80

Ile Ile Asn Met Asp Pro Pro Lys His Thr Lys Ile Arg Ser Val Val

85

90

95

Asn Lys Ala Phe Thr Pro Arg Ala Met Lys Gln Trp Glu Pro Arg Ile

100

105

110

Gln Glu Ile Thr Asp Glu Leu Ile Gln Lys Phe Gln Gly Arg Ser Glu

115

120

125

Phe Asp Leu Val His Asp Phe Ser Tyr Pro Leu Pro Val Ile Val Ile

130

135

140

Ser Glu Leu Leu Gly Val Pro Ser Ala His Met Glu Gln Phe Lys Ala

145

150

155

160

Trp Ser Asp Leu Leu Val Ser Thr Pro Lys Asp Lys Ser Glu Glu Ala

165

170

175

Glu Lys Ala Phe Leu Glu Glu Arg Asp Lys Cys Glu Glu Glu Leu Ala

180

185

190

Ala Phe Phe Ala Gly Ile Ile Glu Glu Lys Arg Asn Lys Pro Glu Gln

195

200

205

Asp Ile Ile Ser Ile Leu Val Glu Ala Glu Glu Thr Gly Glu Lys Leu

210

215

220

Ser Gly Glu Glu Leu Ile Pro Leu Cys Thr Leu Leu Leu Val Ala Gly

225

230

235

240

Asn Glu Thr Thr Thr Asn Leu Ile Ser Asn Ala Met Tyr Ser Ile Leu

245

250

255

Glu Thr Pro Gly Val Tyr Glu Glu Leu Arg Ser His Pro Glu Leu Met

260

265

270

Pro Gln Ala Val Glu Glu Ala Leu Arg Phe Arg Ala Pro Ala Pro Val

275

280

285

Leu Arg Arg Ile Ala Lys Arg Asp Thr Glu Ile Gly Gly His Leu Ile

290

295

300

Lys Glu Gly Asp Met Val Leu Ala Phe Val Ala Ser Ala Asn Arg Asp

305

310

315

320

Glu Ala Lys Phe Asp Arg Pro His Met Phe Asp Ile Arg Arg His Pro

325

330

335

Asn Pro His Ile Ala Phe Gly His Gly Ile His Phe Cys Leu Gly Ala

340

345

350

Pro Leu Ala Arg Leu Glu Ala Asn Ile Ala Leu Thr Ser Leu Ile Ser

355

360

365

Ala Phe Pro His Met Glu Cys Val Ser Ile Thr Pro Ile Glu Asn Ser

370

375

380

Val Ile Tyr Gly Leu Lys Ser Phe Arg Val Lys Met

385

390

395

&lt;210&gt; 43

&lt;211&gt; 1221

&lt;212&gt; DNA

&lt;213&gt; Bacillus subtilis

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (25)..(1212)

&lt;400&gt; 43

ctcgagtcga ggaggtcgac taat atg aac gtt ctg aac cgc cgt caa gcc 51

Met Asn Val Leu Asn Arg Arg Gln Ala

1

5

ttg cag cga gcg ctg ctc aat ggg aaa aac aaa cag gat gcg tat cat 99

Leu Gln Arg Ala Leu Leu Asn Gly Lys Asn Lys Gln Asp Ala Tyr His

10

15

20

25

ccg ttt cca tgg tat gaa tcg atg aga aag gat gcg cct gtt tcc ttt 147

Pro Phe Pro Trp Tyr Glu Ser Met Arg Lys Asp Ala Pro Val Ser Phe

30

35

40

gat gaa gaa aac caa gtg tgg agc gtt ttt ctt tat gat gat gtc aaa 195

Asp Glu Glu Asn Gln Val Trp Ser Val Phe Leu Tyr Asp Asp Val Lys

45

50

55



aaa gtt gtt ggg gat aaa gag ttg ttt tcc agt tgc atg ccg cag cag 243

Lys Val Val Gly Asp Lys Glu Leu Phe Ser Ser Cys Met Pro Gln Gln

60

65

70

aca agc tct att gga aat tcc atc att aac atg gac ccg ccg aag cat 291

Thr Ser Ser Ile Gly Asn Ser Ile Ile Asn Met Asp Pro Pro Lys His

75

80

85

aca aaa atc cgt tca gtc gtg aac aaa gcc ttt act ccg cgc gtg atg 339

Thr Lys Ile Arg Ser Val Val Asn Lys Ala Phe Thr Pro Arg Val Met

90

95

100

105

aag caa tgg gaa ccg aga att caa gaa atc aca gat gaa ctg att caa 387

Lys Gln Trp Glu Pro Arg Ile Gln Glu Ile Thr Asp Glu Leu Ile Gln

110

115

120

aaa ttt cag ggg cgc agt gag ttt gac ctt gtt cac gat ttt tca tac 435

Lys Phe Gln Gly Arg Ser Glu Phe Asp Leu Val His Asp Phe Ser Tyr

125

130

135

ccg ctt ccg gtt att gtg ata tct gag ctg ctg gga gtg cct tca gcg 483

Pro Leu Pro Val Ile Val Ile Ser Glu Leu Leu Gly Val Pro Ser Ala

140

145

150

cat atg gaa cag ttt aaa gca tgg tct gat ctt ctg gtc agt aca ccg 531

His Met Glu Gln Phe Lys Ala Trp Ser Asp Leu Leu Val Ser Thr Pro

155

160

165

aag gat aaa agt gaa gaa gct gaa aaa gcc ttt ttg gaa gaa cga gat 579  
 Lys Asp Lys Ser Glu Glu Ala Glu Lys Ala Phe Leu Glu Glu Arg Asp  
 170 175 180 185

aag tgt gag gaa gaa ctg gcc gcg ttt ttt gcc ggc atc ata gaa gaa 627  
 Lys Cys Glu Glu Glu Leu Ala Ala Phe Phe Ala Gly Ile Ile Glu Glu  
 190 195 200

aag cga aac aaa ccg gaa cag gat att att tct att tta gtg gaa gcg 675  
 Lys Arg Asn Lys Pro Glu Gln Asp Ile Ile Ser Ile Leu Val Glu Ala  
 205 210 215

gaa gaa aca ggc gag aag ctg tcc ggt gaa gag ctg att ccg ttt tgc 723  
 Glu Glu Thr Gly Glu Lys Leu Ser Gly Glu Glu Leu Ile Pro Phe Cys  
 220 225 230

acg ctg ctg ctg gtg gcc gga aat gaa acc act aca aac ctg att tca 771  
 Thr Leu Leu Leu Val Ala Gly Asn Glu Thr Thr Thr Asn Leu Ile Ser  
 235 240 245

aat gcg atg tac agc ata tta gaa acg cca ggc gtt tac gag gaa ctg 819  
 Asn Ala Met Tyr Ser Ile Leu Glu Thr Pro Gly Val Tyr Glu Glu Leu  
 250 255 260 265

cgc agc cat cct gaa ctg atg cct cag gca gtg gag gaa gcc ttg cgt 867  
 Arg Ser His Pro Glu Leu Met Pro Gln Ala Val Glu Glu Ala Leu Arg  
 270 275 280

ttc aga gcg ccg gcc ccg gtt ttg agg cgc att gcc aag cgg gat acg 915

Phe Arg Ala Pro Ala Pro Val Leu Arg Arg Ile Ala Lys Arg Asp Thr

285

290

295

gag atc ggg ggg cac ctg att aaa gaa ggt gat atg gtt ttg gcg ttt 963

Glu Ile Gly Gly His Leu Ile Lys Glu Gly Asp Met Val Leu Ala Phe

300

305

310

gtg gca tcg gca aat cgt gat gaa gca aag ttt gac aga ccg cac atg 1011

Val Ala Ser Ala Asn Arg Asp Glu Ala Lys Phe Asp Arg Pro His Met

315

320

325

ttt gat atc cgc cgc cat ccc aat ccg cat att gcg ttt ggc cac ggc 1059

Phe Asp Ile Arg Arg His Pro Asn Pro His Ile Ala Phe Gly His Gly

330

335

340

345

atc cat ttt tgc ctt ggg gcc ccg ctt gcc cgt ctt gaa gca aat atc 1107

Ile His Phe Cys Leu Gly Ala Pro Leu Ala Arg Leu Glu Ala Asn Ile

350

355

360

gcg tta acg tct ttg att tct gct ttt cct cat atg gag tgc gtc agt 1155

Ala Leu Thr Ser Leu Ile Ser Ala Phe Pro His Met Glu Cys Val Ser

365

370

375

atc act ccg att gaa aac agt gtg ata tac gga tta aag agc ttc cgt 1203

Ile Thr Pro Ile Glu Asn Ser Val Ile Tyr Gly Leu Lys Ser Phe Arg

380

385

390

gtg aaa atg taaggatcc

1221

Val Lys Met

395

&lt;210&gt; 44

&lt;211&gt; 1221

&lt;212&gt; DNA

&lt;213&gt; Bacillus subtilis

&lt;221&gt; CDS

&lt;221&gt; (25)..(1212)

&lt;400&gt; 44

ctcgagtcga ggaggtcgac taat atg aac gtt ctg aac cgc cgt caa gcc 51

Met Asn Val Leu Asn Arg Arg Gln Ala

1

5

ttg ccg cga gcg ctg ctc aat ggg aaa aac aaa cag gat gcg tat cat 99

Leu Pro Arg Ala Leu Leu Asn Gly Lys Asn Lys Gln Asp Ala Tyr His

10

15

20

25

ccg ttt cca tgg tat gaa tcg atg aga aag gat gcg cct gtt tcc ttt 147

Pro Phe Pro Trp Tyr Glu Ser Met Arg Lys Asp Ala Pro Val Ser Phe

30

35

40

gat gaa gaa aac caa gtg tgg agc gtt ttt ctt tat gat gat gtc aaa 195

Asp Glu Glu Asn Gln Val Trp Ser Val Phe Leu Tyr Asp Asp Val Lys

45

50

55

aaa gtt gtt ggg gat aaa gag ttg ttt tcc agt tgc atg cgc cag cag 243

Lys Val Val Gly Asp Lys Glu Leu Phe Ser Ser Cys Met Pro Gln Gln

60

65

70

aca agc tct att gga aat tcc atc att agc atg gac ccg ccg aag cat 291

Thr Ser Ser Ile Gly Asn Ser Ile Ile Ser Met Asp Pro Pro Lys His

75

80

85

aca aaa atc cgt tca gtc gtg aac aaa gcc ttt act ccg cgc gcg atg 339

Thr Lys Ile Arg Ser Val Val Asn Lys Ala Phe Thr Pro Arg Ala Met

90

95

100

105

aag caa tgg gaa ccg aga att caa gaa atc aca gat gaa ctg att caa 387

Lys Gln Trp Glu Pro Arg Ile Gln Glu Ile Thr Asp Glu Leu Ile Gln

110

115

120

aaa ttt cag ggg cgc agt gag ttt gac ctt gtt cac gat tat tca tac 435

Lys Phe Gln Gly Arg Ser Glu Phe Asp Leu Val His Asp Tyr Ser Tyr

125

130

135

ccg ctt ccg gtt att gtg ata tct gag ctg ctg gga gtg cct tca gcg 483

Pro Leu Pro Val Ile Val Ile Ser Glu Leu Leu Gly Val Pro Ser Ala

140

145

150

cat atg gaa cag ttt aaa gca tgg tct gat ctt ctg gtc agt aca ccg 531

His Met Glu Gln Phe Lys Ala Trp Ser Asp Leu Leu Val Ser Thr Pro

155

160

165

aag gat aaa agt gaa gaa gct gaa aaa gcc ttt ttg gaa gaa cga gat 579

Lys Asp Lys Ser Glu Glu Ala Glu Lys Ala Phe Leu Glu Glu Arg Asp

170	175	180	185	
aag tgt gag gaa gaa ctg gcc gcg ttt ttt gcc ggc atc ata gaa gaa				627
Lys Cys Glu Glu Glu Leu Ala Ala Phe Phe Ala Gly Ile Ile Glu Glu				
	190	195	200	
aag cga aac aaa ccg gaa cag gat att att tct att tta gtg gaa gcg				675
Lys Arg Asn Lys Pro Glu Gln Asp Ile Ile Ser Ile Leu Val Glu Ala				
	205	210	215	
gaa gaa aca ggc gag aag ctg tcc ggt gaa gag ctg att ccg ttg tgc				723
Glu Glu Thr Gly Glu Lys Leu Ser Gly Glu Glu Leu Ile Pro Leu Cys				
	220	225	230	
acg ctg ctg ctg gtg gcc gga aat gaa acc act aca aac ctg att tca				771
Thr Leu Leu Leu Val Ala Gly Asn Glu Thr Thr Thr Asn Leu Ile Ser				
	235	240	245	
aat gcg atg ttc agc ata tta gaa acg cca ggc gtt tac gag gaa ctg				819
Asn Ala Met Phe Ser Ile Leu Glu Thr Pro Gly Val Tyr Glu Glu Leu				
250	255	260	265	
cgc agc cat cct gaa ctg atg ccc cag gca gtg gag gaa gcc ttg cgt				867
Arg Ser His Pro Glu Leu Met Pro Gln Ala Val Glu Glu Ala Leu Arg				
	270	275	280	
ttc aga gcg ccg gcc ccg gtt ttg agg cgc att gcc aag cgg gat acg				915
Phe Arg Ala Pro Ala Pro Val Leu Arg Arg Ile Ala Lys Arg Asp Thr				
	285	290	295	

gag atc ggg ggg cac ctg att aaa gaa ggt gat acg gtt ttg gcg ttt 963  
 Glu Ile Gly Gly His Leu Ile Lys Glu Gly Asp Thr Val Leu Ala Phe  
 300 305 310

gtg gca tcg gca aat cgt gat gaa gca aag ttt gac aga ccg cac atg 1011  
 Val Ala Ser Ala Asn Arg Asp Glu Ala Lys Phe Asp Arg Pro His Met  
 315 320 325

ttt gat atc cgc cgc cat ccc aat ccg cat att gcg ttt ggc cac ggc 1059  
 Phe Asp Ile Arg Arg His Pro Asn Pro His Ile Ala Phe Gly His Gly  
 330 335 340 345

atc cat ttt tgc ctt ggg gcc ccg ctt gcc cgt ctt gaa gca aat atc 1107  
 Ile His Phe Cys Leu Gly Ala Pro Leu Ala Arg Leu Glu Ala Asn Ile  
 350 355 360

gcg tta acg tct ttg att tct gct ttt cct cat atg gag tgc gtc agt 1155  
 Ala Leu Thr Ser Leu Ile Ser Ala Phe Pro His Met Glu Cys Val Ser  
 365 370 375

atc act ccg att gaa aac agt gtg ata tac gga tta aag agc ttc cgt 1203  
 Ile Thr Pro Ile Glu Asn Ser Val Ile Tyr Gly Leu Lys Ser Phe Arg  
 380 385 390

gtg aaa atg taaggatcc 1221  
 Val Lys Met  
 395

&lt;210&gt; 45

&lt;211&gt; 396

&lt;212&gt; PRT

&lt;213&gt; Bacillus subtilis

&lt;400&gt; 45

Met Asn Val Leu Asn Arg Arg Gln Ala Leu Pro Arg Ala Leu Leu Asn

1

5

10

15

Gly Lys Asn Lys Gln Asp Ala Tyr His Pro Phe Pro Trp Tyr Glu Ser

20

25

30

Met Arg Lys Asp Ala Pro Val Ser Phe Asp Glu Glu Asn Gln Val Trp

35

40

45

Ser Val Phe Leu Tyr Asp Asp Val Lys Lys Val Val Gly Asp Lys Glu

50

55

60

Leu Phe Ser Ser Cys Met Pro Gln Gln Thr Ser Ser Ile Gly Asn Ser

65

70

75

80

Ile Ile Ser Met Asp Pro Pro Lys His Thr Lys Ile Arg Ser Val Val

85

90

95

Asn Lys Ala Phe Thr Pro Arg Ala Met Lys Gln Trp Glu Pro Arg Ile

100

105

110



Gln Glu Ile Thr Asp Glu Leu Ile Gln Lys Phe Gln Gly Arg Ser Glu  
115 120 125

Phe Asp Leu Val His Asp Tyr Ser Tyr Pro Leu Pro Val Ile Val Ile  
130 135 140

Ser Glu Leu Leu Gly Val Pro Ser Ala His Met Glu Gln Phe Lys Ala  
145 150 155 160

Trp Ser Asp Leu Leu Val Ser Thr Pro Lys Asp Lys Ser Glu Glu Ala  
165 170 175

Glu Lys Ala Phe Leu Glu Glu Arg Asp Lys Cys Glu Glu Glu Leu Ala  
180 185 190

Ala Phe Phe Ala Gly Ile Ile Glu Glu Lys Arg Asn Lys Pro Glu Gln  
195 200 205

Asp Ile Ile Ser Ile Leu Val Glu Ala Glu Glu Thr Gly Glu Lys Leu  
210 215 220

Ser Gly Glu Glu Leu Ile Pro Leu Cys Thr Leu Leu Leu Val Ala Gly  
225 230 235 240

Asn Glu Thr Thr Thr Asn Leu Ile Ser Asn Ala Met Phe Ser Ile Leu  
245 250 255

Glu Thr Pro Gly Val Tyr Glu Glu Leu Arg Ser His Pro Glu Leu Met  
260 265 270

Pro Gln Ala Val Glu Glu Ala Leu Arg Phe Arg Ala Pro Ala Pro Val

275

280

285

Leu Arg Arg Ile Ala Lys Arg Asp Thr Glu Ile Gly Gly His Leu Ile

290

295

300

Lys Glu Gly Asp Thr Val Leu Ala Phe Val Ala Ser Ala Asn Arg Asp

305

310

315

320

Glu Ala Lys Phe Asp Arg Pro His Met Phe Asp Ile Arg Arg His Pro

325

330

335

Asn Pro His Ile Ala Phe Gly His Gly Ile His Phe Cys Leu Gly Ala

340

345

350

Pro Leu Ala Arg Leu Glu Ala Asn Ile Ala Leu Thr Ser Leu Ile Ser

355

360

365

Ala Phe Pro His Met Glu Cys Val Ser Ile Thr Pro Ile Glu Asn Ser

370

375

380

Val Ile Tyr Gly Leu Lys Ser Phe Arg Val Lys Met

385

390

395